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Evolution of HEp-2 cells under the influence of a chronic infection of Oral Polio Vaccine

(Submitted by academician K.G. Karageuzyan 13/VI 2003)

Chronically infected cell cultures are a long-term associations between a host cells and viruses, characterized by long coexistence of a virus and cell. The preservation of the viability of infected cells during persistence is caused by the interaction of various viral and cellular factors. During chronic viral infection a virus or cell or both are changed. The evolution occurs through selection of more resistant population of cells or less cytopathogenic virus [1]. Poliovirus can persistently infect some human cell lines in vitro. Arising in vitro conditions of a chronic virus infection by picornaviruses, and in particular by the poliomyelitis virus, is caused by the appearance of resistant cell clones [2]. In such cells a virus is replicated in lower titers compared to control cell without causing destructive changes. The point of interest is the study of proliferative potential changes in cancer cell cultures and degree of cells differentiation under the influence of chronic virus infection.

Such parameters as the ploidy, size and the quantity of nuclei and nucleoli are a valuable diagnostic attributes of the transformed cells proliferation speed. [3,4,5].

Materials and methods. Cells. In work we used the transformed continuous cell culture of a human larynx cancer - HEp-2. Cells were cultivated in Eagle medium with glutamine and 10% bovine serum. A monolayer of the intact cells was used in 48 hours after the passage. Cells were resowed in dose 1×105 cell/ml. The cell lines were received from laboratory U 322 INSERM "Retrovirus et maladies associeees" Marseilles. The choice of HEp-2 as a model is motivated by the facts from publications on susceptibility of these cells to the Sabin poliovirus strains.

Virus. In work was used the standard trivalent Oral Polio Vaccine (OPV) (Polio Sabin^{TM} [oral] Poliomyelitis vaccine, live attenuated SB BIOLOGICALS Rixensart - BELGIUM).

Chronic virus infection was received by one time infection of 48-hour monolayers of culture HEp-2 by OPV. The multiplicity of infection was 0,000001 TCD_{50} per cell. Infected cells were incubated at 36,5-37^{0}C. Persistent infection was repeated 3 times and summarized data were presented. Viral titer was calculated by the method of Karber. As a control the parallel conducted passages of noninfected HEp-2 culture were used. Their summarized data are given in the table.

Quantitation of nuclear DNA. For the analysis of the received data, the cells preparations of HEp-2 culture were fixed in 96^0 ethyl alcohol for 30 minutes and stained in fresh Shiffs reactive, by Feulgen. The content of DNA in a nucleus and nucleolus was defined by means of computer-equipped microscope-photometer SMP 05 (OPTON). The television method was used on 575 nm waves. In each case 50 - 100 cells were measured.

The DNA content is expressed in a "c" scale in which 1c is half (haploid) the mean nuclear DNA

content of cells from a normal (non-pathological) diploid population in G_0/G_1 cell cycle phase. The DNA image cytometric measurements identified cell nucleuses as aneuploid if they deviate more than 10% from the 2c, 4c, 8c, 16 c, i.e. if they are outside 2c±0,2, 4c±0,4, 8c±0,8, 16c±1,6. The number of all cells in euploid regions of the DNA histogram rescaled by the mean corrective factor of the tissue type under investigation (1.8c - 2.2c; 3.6c - 4.4c; 7.2c - 8.8c; 14.4-17.6) also was calculated [6].

Statistical analysis. For comparison of two groups the non-parametric U test analysis according to Mann-Whitney was applied. Also statistical analyses were performed with Student's t test in the SPSS version 8.0-computer software package (SPSS, Inc., Chicago, IL).

Results and discussion. After the infection, during 2-3 passages of OPV in cell culture, cytopathogenic action of a virus was found as the degradation of the monolayer. However, destruction of a monolayer was not complete, and after the 3rd passage the cytopathogenic action of the virus was reduced. Received chronically infected culture was characterized by insular and slower growth (approximately twice), lack of capacity to form monolayer and constant allocation of a virus. During 2nd 3rd passages, cytopathogenic action of a virus was found. After the 3rd passage the reduction of cytopathogenic action of a virus was observed. The virus was found from the 1, 2, 3, 5, 9 passages in cell-free medium in titers 1,5-0,5 lg/ml. The highest titer was observed only once at 3-th passage (1,75). During the further passages the virus was found out only in cells after their destruction by freezing in low titres (0,25-0,75). A virus accumulated on sensitive intact cell culture HEp-2. So, low viral titres may be explained as a result of decreased quantity of the sensitive cells in infected culture, and in first passages as results of low multiplicity of infection. Quantity of the cells in all passages after 2 was significantly less in comparison with control.

So in the 1-st passage, we can observe the significant growth in the number of cells with 4 nucleoli, as well as the tendency to increase in the percentage of cells with 5 nucleoli (0,72±0,08). This happened first of all due to significant decrease in the number of cells with 1 nucleolus. From the 2-nd passage the number of 4 nucleolar cells decreases and at the same time the quantity of cells with 1 nucleolus increases. These processes continue and by the 5-th passage the number of cells with 4 nucleoli sharply decreases, cells with 5 nucleoli almost disappear (in following passages they percent vary from 0,07±0,01 up to 0,16±0,03). In comparison with the control and the 1-st passage the quantity of cells with 1 nucleoli significantly raised. From 6-th passage the quantity of nonnucleolar cells significantly increases, at practically the same parameters of cells of other types. By the 12-th passage all these parameters almost do not changes. Only the number of nonnucleolar cells in relation to the 6-th passage increases. In this passage in relation to the control a significant decrease in the number of 4 nucleolar cells and increase in the quantity of 1 nucleolar cells is observed. In all the passages significantly increased the number of dead cells, and by 12-th passage the quantity of mitoses significantly decreased. From the 6-th passage appeared significant number in comparison with the control of nonnucleolar cells, the number of which continued to increase, and by the 12-th passage it was significant not only in relation to the control, but also to the 6-th passage. At the same time the number of dead cells significantly increased and the quantity of mitoses decreases (except the 5-th passage). Significant changes in the quantity 2- and 3- nucleolar cells were not fixed in any passage in comparison with the control.

Table 1

Nuclear DNA quantity in HEp-2 cells under the influence of chronic viral infection

Passage	control	1	5	6	9	12
quantity on DNA (in conventional units)	178.88 ±12.39	179.31 ±19.92	165.46 ±18.15	164.61 ±22.51	134.72 ±19.44	137.12 ±17.25*

*Significant in comparison with the control $p < 0,05$

The received data testified that: under the influence of a chronic viral functional activity of the tumour cells in culture is decreased and in the greater degree the characteristics of their differentiation were changed. The results of the study testify that the proliferative activity of HEp-2 cells was decreased under the influence of a chronic viral infection.

Changes in DNA-ploidy were directly related to the differentiation stage and cell proliferation that is why the quantity of DNA in interphase nucleuses of HEp-2 cell line (table 1) was also investigated. We showed, that by the 12-th passage, under the influence of OPV this DNA parameter significant decrease in nuclei of cells of a line HEp-2.

We also investigated the quantity of DNA in cells of each passage with various quantities of nucleoli. In our work it was not revealed significant changes in the quantity DNA in cells with various quantity of nucleoli. Thus, fluctuations of quantity of the nucleolar organizers were not connected with the change of quantity of the DNA in the nucleus.

It was also investigated the area of nucleoli, and difference in nucleoli between 1- 2 -, 3 -, 4 -, 5-nucleolar cells and DNA quantity in nucleoli. In works [7] were showed that area of nucleoli have a strong correlation with the changes of the cellular population doubling time, so and with proliferation activity. It is known, that the correlation of the number both size nucleolar organizer regions and quantity, size and form of the nucleolus was determined by quantity, or by the transcriptional activity of ribosomal DNA.

Table 2

Changes of nucleolar DNA quantity and nucleolus square in HEp-2 cells under the influence of chronic viral infection

Pas-	1 nucleolus		2 nucleolus		3 nucleolus		4 nucleolus	
sage	DNA quantity	square	DNA quantity	square	DNA quantity	square	DNA quantity	square
1	19.9±2.7*	7±1	23.4±4.9	9±2	27.4±7.1	10±3	35±2.7**	14±4
5	20.3±4.3*	7±2	24.4±3.6	9±2	29.7±4.7	11±2	26.1±4.2*	11±1
6	21.8±3.8	8±2	24.9±3.9	9±2	33.4±7.3	12±3	32.1±4.1	11±3
9	21.5±4	8±2	22.2±6.1	8±2	26.3±4.1	10±2	27.5±5.5	10±2
12	22.7±3.3*	8±2	26.1±5.9	9±2	30.2±8.1	11±3	32.5±4.1	12±1
Cont	28.8±3.9	10±3	27.1±5.1	10±3	29.4±6.1	11±2	37.7±2.3	13±2

*Significant in comparison with the control p < 0,05

**Significant in comparison with the 1 nucleolar cells p < 0,05

Our experiments have shown the reduction of the DNA quantity in the nucleoli of mononucleolar cells in comparison with the control after the beginning of influence of the chronic viral infection (tab. 2). This parameter is significant in the 1st, 5th, 7th and 12th passages. Other passages show only a tendency to the decrease of the DNA quantity. The study of 4 nucleolar cells resulted in the fact that a significant decrease of the DNA quantity in the nucleoli was observed only once in the 5th passages. Other passages show only a tendency to the decrease of DNA quantity in the nucleoli.

Comparing mononucleolar and 4 nucleolar cells of the same passage, we conclude that there is no significant difference in the quantity of DNA of mononucleolar cells and total DNA of 4 nucleolar cells. There were no significant changes in the 2 and 3 nucleolar cells either in comparison with the control or inside each passage. The study of the nucleolar area of the HEp-2 cells showed a tendency to the increase of the total nucleolar area in the 4 nucleolar cells.

All mentioned above testifies to the change of nucleolar parameters of mononucleolar cells in the infected cells affected by the chronic viral infection. This enables us to put forward the genetically difference of these cells from the controlled ones.

The study of the nucleolar area revealed a tendency to the increase of total nucleolar area in 4 nucleolar cells in comparison with mononucleolar one, excluding the 1st passage where the increase of the total DNA quantity is significant but in other cases only the tendency (t varies from 0,88 to 1,94).

Distribution by the DNA ploidy and percentage of the euploid cells also was studied. As noted in table 5 the increasing of the number of euploid cells was observed at chronic infection. So, ploidy balance (difference between the percentages of euploid and aneuploid cells) was decreased. In table 3 shown the significant reduction of DNA ploidy during chronic viral infection in HEp-2 cells which was observed since 9th passage.

Table 3

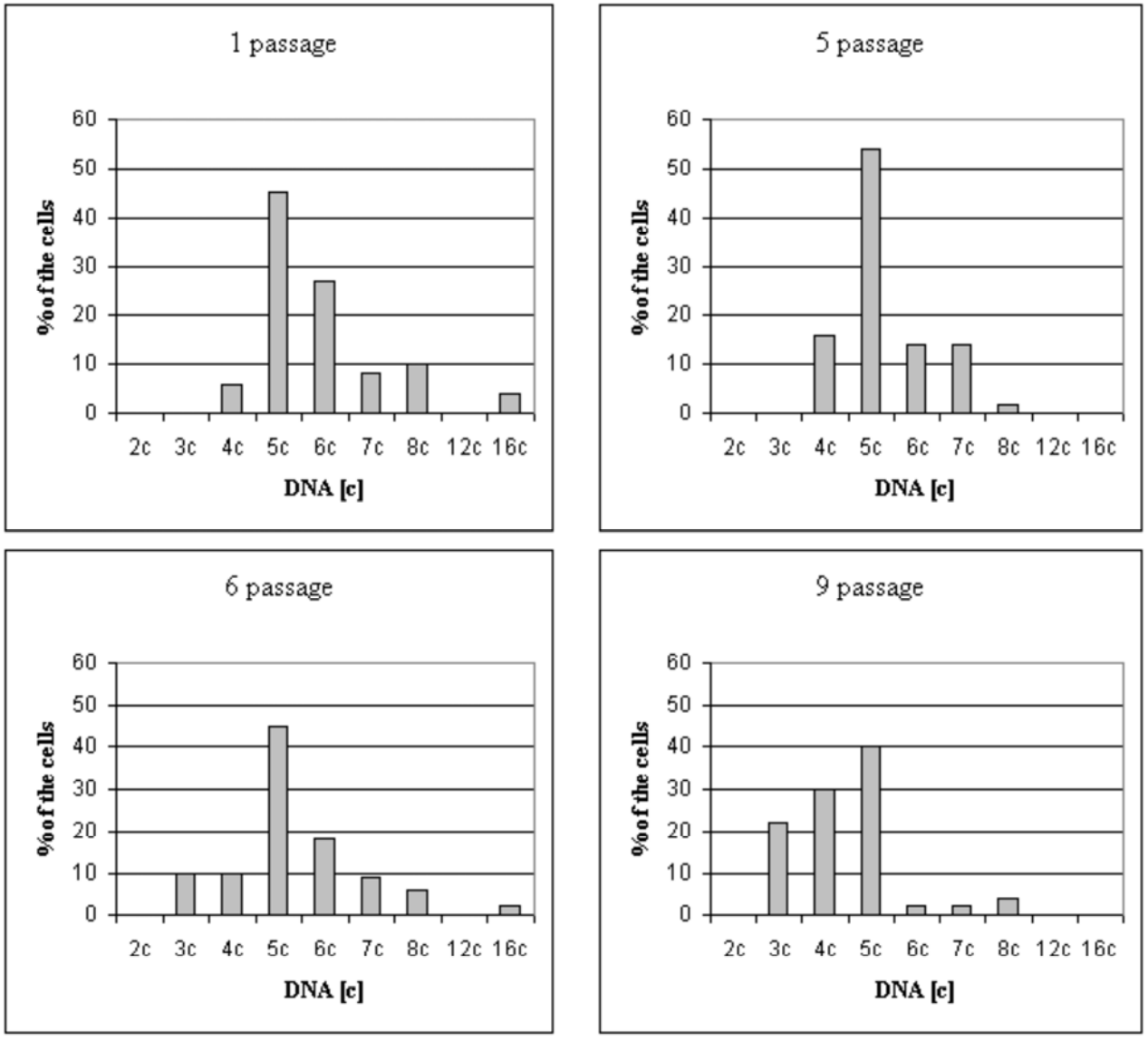
Changes in DNA ploidy in HEp-2 cells during chronic viral infection

	Passages					
	control	1	5	6	9	12
% of the euploid cells	12±3.1	20±3.9	18±5.2	18±4.5	34±7.1	34±5.3*
Average quantity of the DNA in "c" units	5.96	5.98	5.52	5.49	4.51	4.57

*Significant in comparison with control and 1, 5, 6 passages

Fig 1 summarizes changes in DNA ploidy indices during chronic viral infection. The present results indicate that under the influence of OPV is decreased average ploidy of HEp-2 cells. The first time the essential quantities of the diploid cells increases in 12th passage. Percentage of aneuploid cells was decreased in 9 and 12 passages in comparison with the control.

The present results indicate that there are significant differences in various nuclear and nucleolar indices in HEp-2 cells during chronic viral infection. Summing up, at 9 - 12 passages there is an increase in the quantity of mononucleolar cells and accordingly decreases in that of 4 nucleolar ones and stabilization in the quantity of 2 and 3 nucleolar cells. There is also a significant decrease in the percentage of mitosis and increase in the percentage of dead cells.



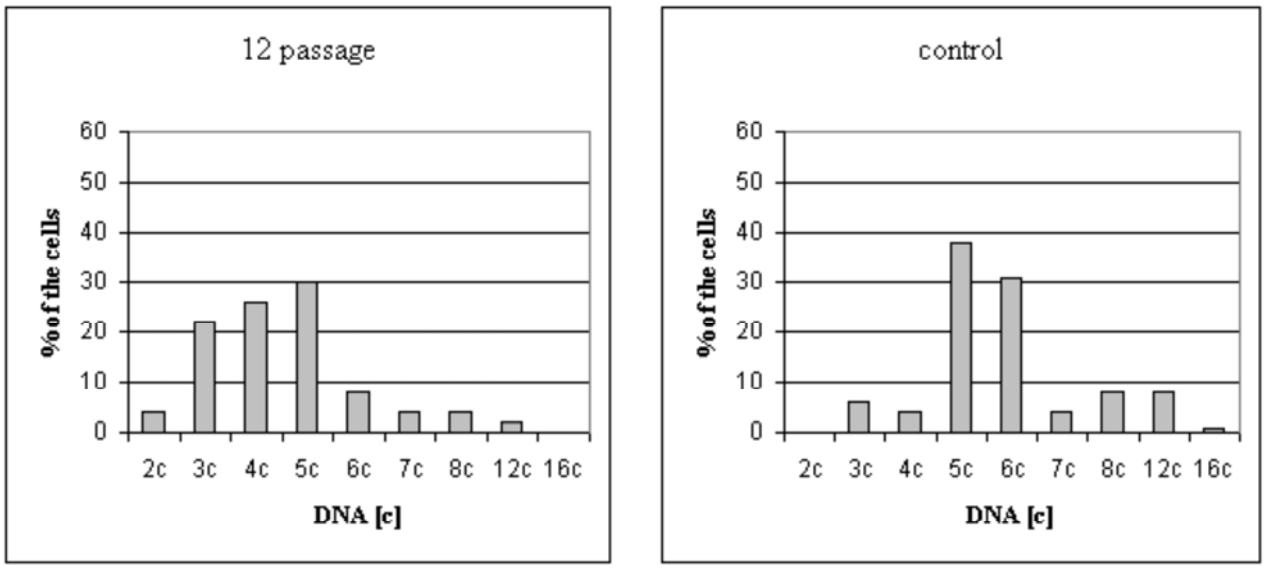


Figure 1. Distribution of the nucleus by the DNA ploidy (in "c" units) in HEp-2 cells during chronic viral infection (shown only a significant local maximum in the DNA histogram)

The evolution of cell population during chronic viral infection in vitro condition takes place through selection of more resistant population of cells or less cytopathogenic virus. Viability of infected cells in vitro during persistence is caused by the interaction of various viral and cellular factors. In our research the chronic viral infection could be the result of action as cellular as viral factors because of in the literature were presented data about unstable Sabin strains of poliomyelitis at 37^0C. However, the used virus keep citotoxic effect on sensitive cells. In the other hand, in our experiment was shown significant difference between intact cells and cells of 9-12 passages. Proliferative activity in this population was sharply decreased. The chronically infected cells had a decreased ploidy index and significantly increased number of the cells with euploid quantity of the DNA in nucleus in comparison with control. These data allow to assume, that under the influence of OPV there was a decrease of a proliferation activity of HEp-2 cells and increase their differentiation [6].

The action of this factor was probably the main reason of the change in nucleolar and nuclear parameters of the HEp-2 culture. It is known that the number of the nucleolar-forming regions in cells is realized genotypically [8]. So, the changes in their quantity give us possiblity to assume that the occurrence of more differentiated and less active proliferating clones of cells is conditioned by the selective cytodestruction of the less differentiated cells during the chronic viral infection. This conclusion is based on the data that various mutations including virus-induces are more dangerous for active divided cells, than for less active. This supposition is made true by the significant changes of the nucleolar parameters (the DNA quantity in nucleoli) and the increasing of the quantity of euploid cells at comparing the control of the HEp-2 cell with the affected cells by chronic infection of the OPV.

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Զ. Ա. Կարալյան

HEp-2 բջիջների էվոլուցիան օրալ պոլիովակցինայի քրոնիկ ինֆեկցիայի ազդեցության ներքո

Մեր կողմից ուսումնասիրվել է HEp-2 բջիջների փոփոխությունները եռավալենտ օրալ պոլիոմիելիտային վակցինայի (Sabin) քրոնիկ ինֆեկցիայի ազդեցության տակ: Պերսիստենցիայի ընթացքում դիտվել է անկորիզակային բջիջների առաջացում և 1 կորիզակային բջիջների տոկոսի աճ; իսկ 4 կորիզակային բջիջների նվազում: Այս ամենը տեղի է ունեցել 2 և 3 կորիզակային բջիջների քանակի կայունացման պայմաններում: Կուլտուրայում նվազել է միտոզների քանակը, իսկ մեռած բջիջների քանակը՝ աճել: Պերսիստենցիայի 12-րդ պասաժում HEp-2 բջիջներում ԴՆԹ-ի քանակը զգալի նվազել է: Քրոնիկ ինֆեկցված բջիջները ունեցել են ցածր պլոիդության ցուցանիշներ: Ինտակտ կուլտուրայի համեմատությամբ բարձրացել է էուպլոիդ բջիջների քանակը: Ստացված տվյալների համաձայն մենք կարող ենք ենթադրել, որ HEp-2 բջիջներում քրոնիկ վիրուսային ինֆեկցիան առաջ է բերում բջիջների պրոլիֆերացիայի նվազում և բարձրանում է նրանց դիֆերենցիացիան:

З.А. Каралян

Эволюция клеток HEp-2 под действием хронической инфекции оральной полиовакцины

Нами исследованы изменения в клетках HEp-2 под действием хронической инфекции трехвалентной оральной полиомиелитной вакцины (Sabin). В процессе персистенции нами наблюдалось появление безядрышковых клеток и возростание процента 1 ядрышковых клеток, а процент клеток с 4 ядрышками снижался. Все это происходило при стабилизации количества 2 и 3 ядрышковых клеток. В культуре уменьшалось количество митозов, а количество мертвых клеток возросло. На 12-ом пассаже персистенции в ядрах HEp-2 количество ДНК значительно уменьшилось. Хронически инфицированные клетки имели сниженный индекс плоидности. Количество эуплоидных клеток возросло по сравнению с интактной культурой. На основе полученных данных мы можем предположить, что хроническая вирусная инфекция в клетках HEp-2 вызывает угнетение пролиферации клеток и увеличивает их дифференцировку.