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CHARACTERIZATION OF THE CONTINUOUS GREEN MONKEY SPLEEN CELL LINE 455

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Summary. — A continuous line of adult green monkey spleen cells has been established and designated 455 (according to the number of animal). Cell suspension for primary explantation has been prepared by perfusion and disaggregation of the organ. The obtained culture, which consisted of fibroblast-like cells with chromosome modal number 60, underwent 25 passages followed by ageing and death (line 455 D). At passage level 13 in 2 culture flasks with 455 D cells, islets of polygonal cells had developed within 35 days; they gave rise to a continuous cell line with chromosome modal number 110 (designated line 455). Line 455 has a high proliferative activity but low demands on the composition of culture media and it can be easily passaged with the bovine serum at concentration of 1—5%. It is highly sensitive to a number of viruses. It is not contaminated with bacteria, fungi, mycoplasms or viruses, it does not have a tumourigenic activity. Hence, it is a promising cellular model for virological studies, in particular, for preparation of inactivated vaccines along with Vero and 4647 cells. The line 455 can be used for investigation of the problems of haemopoiesis and immunogenesis.

Key words: *green monkey*; *line 455*; *spleen perfusion*

Introduction

The design, of suitable cellular models of which biologists and physicians are permanently in need, is of practical and theoretical importance. In recent decades, a large experience has been gained at work with different types of cultures — primary, diploid and heteroploid ones. At present especially important are heteroploid cell lines capable of unlimited subcultivation. In particular, green monkey cell lines Vero and 4647 have been used for the preparation of antiviral vaccines (Menetrat *et al.*, 1981; Mironova *et al.*, 1981; 1982).

The present paper deals with morphological, biological, and genetic properties of the established cell line of adult green monkey spleen designated

455 according to animal number. It has been shown that this cellular system can be properly used as substrate for virus reproduction, including vaccine strains, as well as for the study of haemopoiesis.

Materials nad Methods

Spleen of adult green monkey was disaggregated by perfusion using 0.02% versene and 0.25% trypsin solutions at 37 °C. To prepare primary cultures, the cell suspension was inoculated into culture flasks at 4×10^5 cell in 1 ml. Growths medium consisted of equal amounts of Eagle's medium and 0.5% lactalbumine hydrolysate in Hanks' solution (LAHHE) pH 7.0—7.2 with addition of 10% calf serum. During subcultivation the cells were removed from glass with a mixture of 0.25% trypsin and 0.02% versene solution (2 : 1). Eagle's MEM with 2—5% bovine serum (BS) served as growth medium.

For storage of cells in liquid nitrogen mixture consisting of 80% Eagle's MEM, 10% of glycerine and 10% of BS has been used. The cells were frozen under conventional conditions.

Differential staining of sister chromatids was caried out as described by Chebotarev *et al.* (1972), the number of sister chromatid exchanges (SCE) has been determined.

Isoenzymes glucoso-6-phosphate dehydrogenase (G-6-PDH) and lactate dehydrogenase (LDH) were estimated in cells by the method of Tsareva *et al.* (1975).

The control of the cell line 455 for contamination with alien agents and for tumourigenic activity was conducted in animals and cell cultures to meet the W.H.O. requirements to human diploid cells used for preparation of vaccines in the Laboratory for Improvement of Methods of Vaccine Control (Institute of Poliomyelitis and Viral Encephalitides, U.S.S.R. Academy of Medical Sciences)[1]. The culture was tested for mycoplasms using the method of Chen (1977). Cell cultivation in semiliquid agar was conducted according to MacPherson and Montagnier (1964). Sensitivity of the cell line 455 to viruses was estimated by conventional methods. Statistical treatment was performed according to Strelkov (1966).

Results

After primary explantation of spleen cells from adult green monkey 455, the monolayer had formed on cultivation days 12—14. In the course of subcultivation before the natural death of the cells 3 growth phases have been observed: formation at passages 1—4, active growth at passages 5—20, ageing and death — during passages 21—25. This cell line was designated 455 D. In the active growth phase at passage level 14 and 15, the number of population duplications and the population duplication time were 1.5 and 44 hr, respectively.

At passage level 13 the cells of line 455 D began to die in two flasks. After 35 days islets of polygonal cells had been detected which gave rise to a continuous cell line designated line 455. The cells of the line 455 underwent 157 passages (time of observation). The monolayer formation during the passages in ratios 1 : 2—1 : 4 lasted for 24 to 72 hr, respectively (inoculation dose $6.0—3.0 \times 10^4$ cells in 1 ml). The cells of the line 455 grew well in different growth media with 2—5% of BS. Maximal quantity of cells per 1 cm^2 was $100 . 1 \pm 7.9 \times 10^3$ in LAHHE medium, minimal quantity was $48.4 \pm \pm 4.4 \times 10^3$ in RK-5 medium (modified medium 199). This yield means a 7-8-fold and 3-4-fold increase of cells, respectively. The number and time of population duplications between passages 37, 38 and 111, 112 were virtually

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Table 1. Characterization of line 455 cells at passage levels 37 (A) and 111 (B) (M ± SD)

Growth medium	BS (%)	Plating efficiency (after 6 hr)		Maximum density of cells per 1 cm² (× 10³)		No. of population duplications		Population duplication (hr)	
		A	B	A	B	A	B	A	B
Eagle's MEM	2	70.2 ± 2.4	71.2 ± 2.6	81.8 ± 6.3	86.8 ± 5.3	2.7 ± 0.3	2.8 ± 0.2	44.8 ± 2.6	43.2 ± 2.2
The same	5	77.3 ± 3.5	76.4 ± 2.1	81.4 ± 7.0	84.1 ± 4.7	2.7 ± 0.4	2.7 ± 0.3	44.8 ± 3.6	43.6 ± 3.1
The same	10	83.3 ± 3.1	84.0 ± 3.6	66.4 ± 6.2	67.4 ± 5.6	2.4 ± 0.2	2.4 ± 0.2	49.6 ± 2.1	49.6 ± 2.5
The same	20	83.2 ± 2.6	83.7 ± 2.8	70.5 ± 6.8	69.9 ± 6.3	2.5 ± 0.3	2.5 ± 0.3	47.6 ± 1.8	47.6 ± 2.0
LAHHE	5	80.4 ± 2.7	81.1 ± 3.3	97.0 ± 7.8	100.1 ± 7.7	2.9 ± 0.3	3.0 ± 0.2	32.5 ± 1.5	31.8 ± 1.4
Eagle	5	75.2 ± 2.7	73.7 ± 3.1	81.5 ± 5.8	81.5 ± 5.6	2.7 ± 0.2	2.7 ± 0.2	44.8 ± 3.4	44.8 ± 3.1
Eagle's MEM with 2nd set of amino acids and vitamins	5	76.6 ± 3.4	78.5 ± 2.7	81.7 ± 6.0	81.5 ± 5.6	2.7 ± 0.2	2.7 ± 0.2	44.8 ± 2.6	44.8 ± 2.3
Medium 199	5	73.6 ± 2.8	75.2 ± 3.1	68.0 ± 0.4	68.6 ± 4.8	2.4 ± 0.2	2.4 ± 0.3	48.9 ± 3.6	48.9 ± 3.3
Medium RK-5	5	73.6 ± 2.8	75.2 ± 3.1	68.0 ± 0.4	68.6 ± 4.3	1.9 ± 0.2	1.9 ± 0.2	61.5 ± 3.8	61.5 ± 3.0

Note. Inoculation dose was (12.5 ± 0.7) × 10³ cells/cm² in all variants.

the same and amounted to 1.9 and 61.5 hr in RK-5 medium and 3.0 and 32.5 hr in LAHHE medium, respectively. The data obtained during cultivation of cell line 455 in other media are presented in Table 1.

After storage in liquid nitrogen for 1.5—2.5 years the cells of line 455 retained their original properties, and 92.1 $\pm$ 2.7% of cells remained viable.

Morphological study has demonstrated that line 455 D basically consists of fibroblast-like cells (at early passages — passage 1 to 5 — polygonal, spindle-like and round-shaped cells occurred. At the phase of ageing and death the cells acquired a polygonal shape, the number of hypertrophic cells with granular cytoplasm had increased. Line 455 consisted of polygonal cells with bulky nuclei containing 1—3, rarely 4 nucleoli. Two basic varieties of cells predominated — elongated spindle-like and round-shaped cells (Fig. 1).

The analysis of metaphase plates of cell line 455 D at different passages has revealed modal chromosome number equal to 60, karyotype was the same as that of male African green monkey (2 n = 60, XY). Percentage of diploid cells ranged between 78.4 and 82.1, that of hypoploid cells ranged from 1.3 to 2.1, and of hyperploid cells from 14.8 to 15.3, the polyploid cells made 8.4—9.0% of the latter.

In the line 455 cells the modal chromosome number was 110, both at early and late passages. The percentage of tetraploid cells ranged from 36.0 to 40.2, that of hyperploids from 58.2 to 62.7, and of diploids from 4.0 to 6.3. Frequency of SCE varied from 4 to 19. Exchanges were observed in different chromosome groups (Fig. 2). It has been found that the character of cell distribution with respect to SCE frequency can be described by log of normal distribution. Mean geometric frequency of SCE per cell was 9.76 with upper and lower confidence limits of 8.84 and 10.77, respectively.

The cells of line 455 at passages 45 and 97 possessed glucoso-6-phosphate dehydrogenase activity with type A mobility and lactate dehydrogenase (5 isoforms) characteristic for green monkey cells.

Cell line 455 appeared sensitive to infection with vaccine strains of poliomyelitis virus (Sabini strains): type I — $10^{7.6\pm0.2}$ PFU/ml; type II — $10^{7.5\pm0.2}$ PFU/ml; type III — $10^{7.7\pm0.2}$ PFU/ml; measles virus (strain EShCh) — — $10^{4.5\pm0.2}$ PFU/ml; canine distemper virus (strain Rockborne) — $10^{5.4\pm0.2}$ PFU/ml, as well as to following viruses (titres in $TCID_{50}$/ml): ECHO 1 — — $10^{7.2}$; ECHO 11 — $10^{6.0}$; ECHO 12 — $10^{6.0}$; ECHO 19 — $10^{7.4}$; Coxsackie A7 — $10^{7.2}$; Coxsackie A9 — 10^{5}; Coxsackie A14 — 10^{7}; Coxsackie B1 — 10^{6}; Coxsackie B5 — 10^{6}. The titre values were independent of the passage level of cell line 455. Moreover, this line can endure agar layer containing aminopeptide and sodium bicarbonate and have been succesfully used for titration of poliomyelitis and canine distemper viruses by plaque technique. The results obtained in line 455 cells and in primary green monkey cell cultures were essentially identical.

No foreign viruses have been detected by inoculation of the culture fluid of 455 cells into diploid human embryo cell cultures, primary green monkey kidney cell cultures and rabbit kidney cells in two subsequent passages.

Haemadsorption test with guinea pig, sheep, and goose red blood cells was negative. No bacteria or fungi have been detected in 455 cells at any of the passage levels tested. The tests for the presence of foreign viruses and bacteria have been carried out in rabbits, guinea pigs, adult and infant albino mice, and chick embryos. No foreign agents pathogenic for these animals have been detected in the systems tested. Upon inoculation of 455 line cells to mice treated with antithymocyte serum no tumours have been observed in any of recipient animals throughout the observation period. In positive control KV cell recipients the tumours appeared within 12 to 14 days.

The tests for the presence of mycoplasms with the use of fluorescent Hoechst 33258 stain were negative in all samples of line 455 (passage levels 37, 45, 67, 97, 125). During cultivation of the line 455 cells at passage levels 56 and 97 no growth of these cells has been observed over 35 days (observation period) in semiliquid agar. With control HeLa cells growth zones have been detected on cultivation days 12 to 14.

Discussion

Studies in line 455 cells demonstrated that this culture has a high proliferative activity devoid of foreign agents and tumourigenic activity. These findings as well as the high sensitivity to viruses, suggests that line 455 may be used as a safe substrate for production of antiviral vaccines. This is supported by the available data on successful use of heteroploid cell lines for vaccine production (Menetrat *et al.*, 1981; Mironova *et al.*, 1981; Steenis and Wezel, 1981).

In addition to this practical effect, establishment of the continuous cell line 455 is of great theoretical value. A clone with high proliferative activity has been isolated from spleen stromal cells. This has been done already after 13 passages of diploid fibroblasts, when the cell deaths already started in some culture flasks. The isolation of this clone seems to have been promoted by the absence of enzyme treatment of the developed colony, i. e. of the cell clone: the transfer of cells into another culture flask has been carried out mechanically.

Returning to the moment of establishment of the initial line of diploid cells 455 D, it should be emphasized that cell suspension has been prepared with the use of perfusion disagregation in which haemopoietic and lymphoid cells, and macrophages could be damaged by trypsin and partially removed by the flow of fluid. The remaining part of these elements in the primary suspension appeared to have been eliminated during the first 3 to 5 passages, since fibroblasts are the only type of cells that could actively proliferate for a long time and be maintained in the subcultures (Fridenshtein, Luriya, 1980). According to Fridenshtein and Luriya, the content of colony-producing cells of the spleen of guinea pigs, rabbits and mice was 1 per 10^5 cells. No data are available for monkey spleen. The importance of the preparation of the line of continuous cells of spleen stroma is that it can be used in experiments for design of micro-environment during the formation of haemopoietic territories

both in vivo and in vitro. When diploid fibroblasts are introduced into the organism, they bring with them the micro-environment of the haemopoietic organ.

Unfortunately, special identification of cell line 455 has not been carried out. In particular, it has not been revealed, whether the specific fibroblast antigen, which is not found in macrophages, lymphocytes or haemopoietic cells, is present in these cells. Therefore, we can only point out that the morphology of cells in the line 455 is different from line 455 D. In the phase of active growth, line 455 D consisted of rather bulky fibroblast-like cells. In the line 455, in addition to fibroblast-like spindle-shaped cells (though much smaller than in the line 455 D) there were found many polygonal, round-shaped cells similar to epithelial cells. According to Frideshtein (1974) there is no cytogenetic relationship between stromal fibroblasts and reticular cells on one hand and macrophages on the other. In the adult organism, therefore, line 455 can be expected to contain only fibroblast descendants.

Summing up, line 455 can be used as cellular substrate for virus reproduction, as well as a test object for solving various problems of fibroblast differentiation in culture, their regulating effect on haemopoietic and lymphoid cells, their role in the formation of haemopoietic environment, the phenomenon of morphogenetic interaction with haemopoietic cells and differentiation of immunocompetent cells.

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Legends to Figures (Plate XLII):

Fig. 1. Cell line of adult green monkey spleen 455. Passage 150. Day 2 after subcultivation. Native culture. Lens 12.5×, eye-piece 4×.

Fig. 2. Metaphase plate of the cell of line 455. Passage 75. Lens 90×, eye-piece 7.7×.