Banks of Cell Cultures for Biotechnology

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Seeding and working cell banks were created and stored in cell culture collection. The banks were certified in accordance with international and national requirements. Cultures of 293, MT-4, L-68, FECH-16-1, FECH-16-2, 4647, MDCK, CHO TK$^-$, and CHO pE cells were recommended by Medical Immunobiological Preparation Committee for the use in the production of medical immunobiological preparations. The stock is sufficient enough for supplying standard cell material for the production of medical immunobiological preparations over few decades.

Key Words: *cell culture; banks of cell cultures; cell certification*

Diploid and immortalized cell cultures are now widely used for production of medical immunobiological preparations (MIBP). Cell cultures are stable, standard, and allow obtaining great volume of cell material over a short periods. At the same time, the safety of cell cultures is still a matter of concern (they can induce tumor growth or be contaminated). Moreover, spontaneous transformation of cells cannot be excluded [3]. In light of this, WHO established Requirements for cell cultures as substrates for the production of MIBP [12]. Cell producers used for obtaining MIBP should have stable morphology, growth rate, and karyotype, exhibit no oncogenic potential, contain no contaminants, and retain high productivity over specified number of passages. The use of cultures for MIBP manufacturing is based on the seed lot system.

The aim of the present study was creation and certification of seed and working banks of diploid and immortalized cultures of cells used in MIBP production.

MATERIALS AND METHODS

Cell culture. The study was performed on cell cultures from the collection of Vector Research Center of Virology and Biotechnology. The following cell strains were used for creation of banks: 293 (human embryonic kidney; cells transformed with adenovirus-5 DNA), 4647 (African green monkey kidney cells), MDCK (canine kidney cells), MT-4 (co-cultured lymphocytes from heart blood and peripheral blood from patients with T-cell leukemia), CHO TK$^-$ (thymidine kinase-deficient Chinese hamster ovary cells), CHO pE (Chinese hamster ovary cells, producers of recombinant human erythropoetin), L-68 (diploid cells of human embryonic lungs), FECH-16-1 (diploid cells of human embryonic lungs), FECH-16-2 (diploid cells of human embryonic musculocutaneous tissue).

The cells were cultured in Eagle's MEM or RPMI-1640 media (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalites, Russian Academy of Medical Sciences) supplemented with 10% fetal calf serum (Gibco), cryopreserved in the same medium with 10% glycerol (ICN Biomedicals), and stored at -196°C in liquid nitrogen. After cryopreservation, the cultures were reconstituted by the standard method [1]. Seeding density was (1.0-2.0)×10^6 cells/ml, seeding ratio 1:2-1:4, and period of subculturing 3-4 days. For subculturing, the cells were treated with a 1:2 mixture of 0.25% trypsin and 0.02% versen (Vector Research Center of Virology and Biotechnology). MT-4 cells were harvested mechanically (by shaking). The cultures were incubated at 37°C.

Creation of cell banks. For obtaining seed banks, the initial culture was passaged for 5-6 successive

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passages, the cell biomass was produced, and the cell suspension was transferred to ampoules and cryopreserved. Working banks were prepared from one ampoule of the corresponding seeding bank.

Cell cytomorphology. The cells were grown in tubes on coverslips, fixed in ethanol, stained with hematoxylin and eosin, and examined under a light microscope. Cytomorphology, proliferative activity, concentration, and viability were analyzed as described previously [1].

Karyological analysis. The preparations were made as described elsewhere [1]. Structural rearrangements and abnormalities, the presence of marker chromosomes, the relative content of polyploid cells, and modal number of chromosomes (modal class) were evaluated.

Species specificity. Glucose-6-phosphate dehydrogenase and lactate dehydrogenase isoenzymes were assayed by disk-electrophoresis in polyacrylamide gel [1].

Test for sterility. The test for sterility was performed using the microbiological method by direct seeding into thioglycolate medium [1].

Detection of mycoplasma. The cells were seeded into a medium with 0.3% agar prepared on trypsin-treated cattle heart tissue [1], and examined under a luminescent microscope with Hoechst-33258 staining (0.0005%, Sigma) [1] and PCR [10]. Cell culture samples (10^6 cells) were tested by PCR using primers purchased from Biosintez company (GP01 and MGSO for the first round and GP02 and MGSO for the second round). We also used mycoplasma-infected Vero cells (African green monkey kidney, positive control), deionized water (negative control) and molecular weight marker (Fermentas).

Control for the presence of foreign viruses. Tests were performed on animals (adult and suckling mice, guinea pigs, and rabbits) obtained from the nursery of Vector Research Center of Virology and Biotechnology and on chicken embryos and Vero and L-68 cell cultures [1,12].

Oncogenic potential. The method of heterotransplantation of cells to immunosuppressive animals was used [1]. Cultured HeLa cells (human cervical cancer cells) exhibiting oncogenic potenties was used as the positive control. Diploid L-68 cells served as the negative control.

Control for endogenous oncoviruses. Reverse transcriptase assay was used [1,4]. Dilutions of recombinant HIV-1 reverse transcriptase served as the positive control and polymerase buffer as the negative control.

RESULTS

Seeding and working cell culture banks were obtained by serial subculturing of the same cell pool and simultaneously frozen at the same passage. Banks of cell cultures are stored in aliquots in ampoules at -196°C in cell culture collection of Vector Research Center of Virology and Biotechnology.

The banks of cell cultures were certified in accordance with the requirements for cell cultures used in biotechnology [1,12].

It was proven that cell cultures can be stored in liquid nitrogen for a long time. For instance, L-68 cells from the seeding and working banks obtained in 1983 and 1989 are used for many years for Reaferon testing. Cell reconstitution after cryopreservation showed that 80% cells were viable and that the seeding dose, passaging interval, and proliferative activity of cells remained unchanged. Morphology of cell cultures was typical of these strains.

Cell changeability is a serious problem during cell culturing. Therefore we performed long-term culturing of cells from seeding and working banks for determining the passage level recommended for MIBP production. Diploid cells L-68, FECH-16-1, and FECH-16-2 were cultured over 35 passages, MDSK for 48 passages, CHO-TK^- for 30 passages, and 293, 4647, MT-4, and CHO pE for 20 passages. The specified cells had stable biological properties and demonstrated high proliferative activity and viability. Subseeding ratio did not change during passaging and was 1:2 for diploid and 1:4 for immortalized cells.

Karyological analysis of cell cultures is one of the major criteria allowing identification of cell properties. Karyotyping of cell culture banks was performed in the beginning and at the end of the passage level recommended for MIBP production.

Diploid cells L-68, FECH-16-1 and FECH-16-2 were analyzed during passages 14, 18, and 33. The cells had diploid chromosome set, the karyotype corresponded to normal human karyotype, 2n=46. Marker chromosomes were not detected. Clear cut modal class was presented by cells with 46 chromosomes. Polyploid cells were detected in 0.4-3.0% cases, haploid cells and structural rearrangements were absent. The number of cells with chromatide and chromosome breaks did not exceed 1-3%. Cell karyotype during long-term culturing was stable. Karyological characteristics of diploid L-68, FECH-16-1 and FECH-16-2 cells were consistent with the requirements established by the International Committee on Cell Cultures [1,5,12].

Karyotype of 293 cells was analyzed during passages 18 and 38. It represented a derivative of normal human karyotype, 2n=46. The cells were characterized by variations in the number of autosomes and marker chromosomes and the presence of mono-, tri-, and tetrasomy of many autosomes and frequent absence of normal chromosomes 1 and 13. X chromosomes were not changed. Fifteen marker chromosomes were de-

tected. During passage 18, chromosome number varied from 10 to 101, modal number 71. During passage 38, chromosome number varied from 9 to 81, no clear-cut modal number was determined. The relative content of polyploid cells in passage 18 cultures was 1%; no polyploid cells were detected in passage 38 cultures. The culture of 293 cells had a donor karyotype, long-term culturing over 20 successive passages did not affect the incidence of polyploid cells [7].

Culture of MDCK cells was analyzed during passages 41 and 88. The cells had stable karyotype corresponding to normal canine karyotype, 2n=78. They carried 5 marker chromosomes. Polyploid cells were not detected. Modal class during passages 41 and 88 was presented by cells with 85 chromosomes. The karyotype was characterized by the presence of non-rearranged autosomes. Culture of MDCK cells during passages 41 and 88 had stable karyotype with unchanged modal number [8].

Karyological characteristics of CHO pE cells were evaluated during passages 4 and 23. The karyotype was stable and corresponded to normal karyotype of Chinese hamster, 2n=22. The basic chromosome set included chromosome pairs 1, 2, 3, 4, 6, 7, 8, 9, and 10. CHO pE cells were characterized by the presence of 10 marker chromosomes, modal class 33, cultures during passages 4 and 23 contained 4 and 3% polyploid cells. During long-term culturing of CHO pE cells, the modal num-

TABLE 1. Seeding and Working Banks of Cell Cultures

Culture	Application	Bank	Passage No.	Number of ampoules	Cells		Certification institution
					amount in ampoule	viability, %	
293	Production of preparations for tumor patients	Seeding	15	240	5.7×10^6	85	Vector; ISC
		Working	17	241	5.0×10^6	83	
4647	Production of preventive and diagnostic preparations	Seeding	108	257	4.0×10^6	84	Vector; ISC
		Working	112	231	6.0×10^6	86	
MDCK (NBL-2)	Production of preventive and diagnostic preparations	Seeding	39	209	1.0×10^7	90	Vector; ISC
		Working	41	242	5.0×10^6	98	
MT-4	Experiments with HIV	Seeding	41	203	3.2×10^7	97	Vector
		Working	45	250	1.2×10^7	80	
CHO TK$^-$	Gene engineering	Seeding	13	181	5.0×10^6	80	Vector; ISC
		Working	19	232	6.0×10^6	80	
CHO pE	Production of recombinant human erythropoietin	Seeding	22	216	3.0×10^6	90	Vector; ISC
		Working	27	212	2.5×10^6	80	
L-68	Production and control over MIBP	Seeding	12	200	4.0×10^6	90	Vector; ISC
		Working	16	197	1.7×10^6	93	
		Working	17	212	4.5×10^6	90	
FECH-16-1	Replacement therapy	Seeding	11	204	2.0×10^6	84	Vector; ISC
		Working	15	252	1.5×10^6	82	
		Working	18	235	3×10^6	92	
FECH-16-2	Replacement therapy	Seeding	11	312	2.0×10^6	81	Vector; ISC
		Working	15	268	2.5×10^6	80	
		Working	19	233	2.5×10^6	92	

Note. Vector: Vector Research Center of Virology and Biotechnology; ISC: L. A. Tarasevich Institute for Standardization and Control for Biomedical Preparations.

ber of chromosomes and the content of polyploid cells remained unchanged, karyotype was stable [6].

Analysis of CHO TK$^-$ culture during passages 4, 14, 25, and 35 showed that cell karyotype was stable and corresponded to normal Chinese hamster karyotype, 2n=22. There were 11 marker chromosomes, modal index 34 chromosomes, the relative content of polyploid cells was 1.7-1.2%. Each chromosome pair was presented by two homologues, except chromosome pairs 4, 6, and 10 that had single homologue. The basic chromosome set consisted of chromosome pairs 1, 2, 3, 4, 6, 7, 8, 9, and 10. Karyological parameters of CHO TK$^-$ cells evaluated during passages 4, 14, 25, and 35 remained unchanged.

Karyotype of 4647 cells was analyzed during passages 108 and 128. The karyotype represented a derivative of normal African green monkey karyotype, 2n=60 and was characterized by the presence of normal (nonrearranged) autosomes; C2 trisomy was detected. One of X chromosomes was rearranged. There were 6 marker chromosomes. The chromosome number distribution in cells varied: from 17 to 120 during passage 108 (modal number 60) and from 52 to 136 per cell during passage 128 (no definite modal class). Cell of different ploidy were detected. Polyploid cells constituted 20% during passage 108 and 79% during passage 128. Karyotype of 4647 cells was stable during passages 108 and 128 [9].

Karyological characteristics of MT-4 cells were evaluated during passages 5 and 25. The karyotype corresponded to normal human karyotype (2n=46). There were 4 marker chromosomes: marker M1 is stable for all MT-4 cells and markers M2, M3, and M4 are variable. Polyploid cells constituted 1.4%. Modal class 81-82 chromosomes. The basic chromosome set included all chromosomes of the original species. Each chromosome pair was presented by three or four homologues. The exception was chromosome pair 2 presented by two homologues. Sex chromosomes XXYY. The karyotype of MT-4 cells remained stable during culturing for 20 successive passages.

Thus, we determined passage level for cultures of 293, MT-4, L-68, FECH-16-1, FECH-16-2, 4647, MDCK, CHO TK$^-$, and CHO pE cells for their use in the production of MIBP.

Species specificity of cell cultures was confirmed by results of not only karyological analysis, but also studies of electrophoretic mobility of isoenzymes. We found that the amount and mobility of glucose-6-phosphate dehydrogenase and lactate dehydrogenase in 293, MT-4, L-68, FECH-16-1, and FECH-16-2 cells of seeding and working banks corresponded to those in primate cells, in 4647 cells – to those in green monkey cells, in MDCK – to those in canine cells, and in CHO TK$^-$ and CHO pE – to those in Chinese hamster cells.

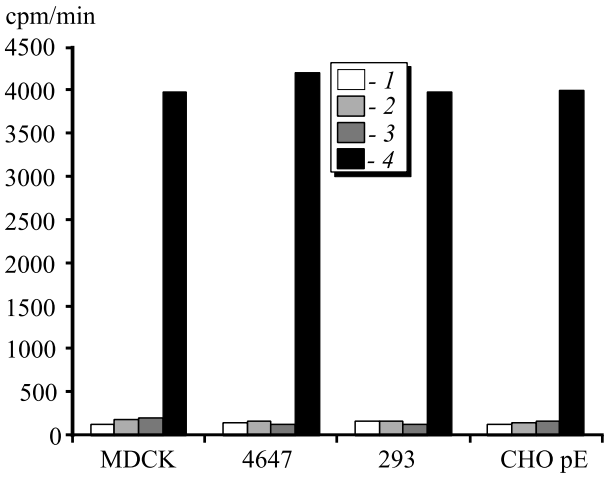

Fig. 1. Reverse transcriptase activity. *1*) seeding cell bank; *2*) working cell bank; *3*) negative control (polymerase buffer); *4*) positive control (sample with 50 ng recombinant HIV reverse transcriptase.

Bacterial, fungal, mycoplasma, and viral contamination is a factor impairing cell stability and limiting their application for MIBP production. Tests for microbial sterility revealed no bacteria and fungi in seeding and working banks of cell cultures.

There are a number of diagnostic tests for mycoplasma detection in cell cultures. Culturing in selective nutrient media is most frequently used. However, not all mycoplasma species can be cultured on selective media, therefore combinations of diagnostic tests should be used. Direct fluorescent staining with luminescent microscopy in combination with DNA staining with intercalating fluorochromes is the most reasonable method for mycoplasma identification in cell culture; it provides stable results and allows mycoplasma detection in cultures even under conditions of low-dose multiple infection [2]. PCR is now also used for mycoplasma detection [10,11]. Tests for the presence of mycoplasma in seeding and working cell banks were performed by microbiological and cytochemical methods and PCR. No mycoplasma colonies were found during culturing of 293, MT-4, L-68, FECH-16-1, FECH-16-2, 4647, MDCK, CHO TK$^-$, and CHO pE cells on special selective media. Hoechst 33258 staining revealed no specific mycoplasma DNA in the cell cytoplasm and extracellular space. PCR confirmed the absence of *Acholeplasma laidlawii*, *M. alkalescens*, *M. arginini*, *M. arthritidis*, *M. bovine*, *M. buccale*, *M. canis*, *M. fermentans*, *M. gallisepticum*, *M. hominis*, *M. hyorhinis*, *M. orale*, *M. pulmonis*, *M. salivarium* in the studied cell banks.

Contamination with latent and chronic viral infection is most difficult to detect [1,12]. Control for the absence of foreign viruses in immortalized Vero cells and diploid L-68 cells detected no cytopathogenic, hemagglutinating, and hemadsorbing latent viruses in

seeding and working cell banks. The results of tests on laboratory animals showed that all inoculated suckling mice survived; autopsy after euthanasia revealed no pathological changes in the viscera. None of the adult animals died after injection of cells from seeding and working banks. In experiments on chicken embryos, more than 80% embryos survived; in none samples of allantoic fluid hemadsorbing agents were detected. Thus, tests showed the absence of viruses inducing morbidity and mortality in animals and chicken embryos in the examined banks of 293, MT-4, L-68, FECH-16-1, FECH-16-2, 4647, MDCK, CHO TK^-, and CHO pE cells.

The absence of oncogenic potencies is a criterion for practical application of cell cultures in MIBP production. The tests with heterotransplantation showed that cells of the studied banks induced no neoplasms, whereas transplantation of HeLa cells (exhibiting potential capacity for tumor growth with metastasizing) induced neoplasms (*e.g.* epidermal carcinoma) in immunosuppressive animals. No pathological changes at the site of transplantation and in the lymph nodes, lungs, kidneys, and liver were detected after inoculation of 293, MT-4, L-68, FECH-16-1, FECH-16-2, 4647, MDCK, CHO TK^-, and CHO pE cells. For verification of these data, reverse transcriptase assay was used intended for detection of endogenous oncoviruses in cells. In the studied cells, the level of reverse transcriptase did not exceed that in the control, which attested to the absence of endogenous oncoviruses in these cells (Fig. 1).

Thus, seeding and working cell banks were created and stored at Vector Research Center of Virology and Biotechnology. The banks were certified at Vector Research Center of Virology and Biotechnology and L. A. Tarasevich Institute for Standardization and Control for Biomedical Preparations (Moscow). Cultures of 293, MT-4, L-68, FECH-16-1, FECH-16-2, 4647, MDCK, CHO TK^-, and CHO pE cells were recommended by Medical Immunobiological Preparation Committee for the use in the production of medical immunobiological preparations. The stock is sufficient enough for supplying standard cell material for the production of medical immunobiological preparations over few decades.

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