A Correlative Study in the Modification of the BHK 21 Cell-line Induced by the Passage Within the Same Genotype

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Introduction

The oncogenic transformation of hamster cells in tissue culture can be produced under the influence of virus (Macpherson and Stoker 1962, Sachs and Medina 1961, Sanford and Hoemann 1967, Stoker and Macpherson 1961, Traykovich) and spontaneously in long term-culture (Defendi *et al.* 1963, Gotlieb-Stematsky and Shilo 1964, Hale *et al.* 1965, Sanford and Hoemann 1967, Stoker and Macpherson 1964, Thomas 1964, Traykovich 1968a). Last phenomenon has been first described in the kidney cells of the new born hamsters (Defendi *et al.* 1963, Gotlieb-Stematsky and Shilo 1964, Hale *et al.* 1965, Stoker and Macpherson 1964, Thomas 1964, Traykovich 1968a), known as continuous line BHK 21 of Macpherson and Stoker (1962), established in May 1961.

Defendi *et al.* in 1963 published about tumorigenic possibility of the BHK 21 cells, and other investigators confirmed this finding (Gotlieb-Stematsky and Shilo 1964, Hale *et al.* 1965, Stoker and Macpherson 1964, Thomas 1964, Traykovich 1968a).

The BHK 21 cells presented in this paper possessed high metastatic quality (Traykovich 1968a), for which they were distinguished from the other BHK 21 tumorigenic lines studied (Defendi *et al.* 1963, Gotlieb-Stematsky and Shilo 1964, Hale *et al.* 1965, Stoker and Macpherson 1964). Aside from this, the cells have demonstrated possibility for heterotransplantation, the growth in recently born mice and rats, as well as on the chorio-allantoic membrane of the chicken eggs (Traykovich 1968b).

The aim of this study is to expose the correlation between the original BHK 21 culture, its development in the hamsters after implantation and its restoration from tumors in tissue culture. The experiment has been divided in three parts:

- A) Characteristics of the original BHK 21 cells;
- B) Behaviour of the cells grafted in the susceptible animals; and
- C) Cellular characteristics after reestablishment of the tissue culture from the tumor.

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Material and methods¹

The BHK 21 cells at the beginning of the experiment were in the 18th laboratoric passage. They were maintained to the instruction of originators (Macpherson and Stoker 1962). The cellular layer was routinely transferred twice a week with glucosated trypsin solution (Tournier 1962).

The cytological study was performed on the cells grown in Leighton tubes, fixed in Boun's fluid and stained with hematoxylin and eosin.

The optimum concentration of cell dose for growth has been simultaneously determinated by the original BHK 21 cell line at the 47th passage, and by the reestablished cell line of intradermal tumoral tissue (BHK 21/TID) at the 30th passage. The following quantity of cells: $5 \times 10^4 - 4 \times 10^5$ /ml were set in six bottles of 2 ozs. During a period of five days a daily inspection was made of cell growth for the formation of complete layer on the surface of bottles, and in the case of acidification of the medium, this has been changed.

The chromosomes were elaborated by the Traykovich and col method (1967b) and colored with Unna stains. The modal (1967b) value has been estimated on the 82 BHK 21 cells, passage 19 and 21, and on the 49 BHK 21/TID cells, passage 46 and 52.

The implantation of the BHK 21 cells in the hamsters were made by the following manner: after trypsinisation and centrifugation on 800 rpm/10 min, they were washed twice in Hanks' fluid. The desired dose was resuspended in the growth medium without serum.

For the orientational test on the tumorigenesis of the BHK 21 cells, the following grafts were made: subcutaneously, intradermal and intraperitoneal in doses of 5×10^6 /ml in three groups of 6 adult animal conditioned with cortisone.

The minimum tumorigen dose of BHK 21 cells were determinated by comparativ mode, intradermal (ID) and subcutaneously (SC) inoculation in the back of hamsters with $1 \times 10^6 - 5 \times 10^7$ /ml of cells in 14 groups of hamsters. Each group containing 6 hamsters, excepting one group of 5 animals.

The serial passages of the BHK 21 tumors were made, making six transplantable generation of the tumor tissue by intradermal and five by subcutaneous mode of grafts. The groups were composed of 6 animals, except in two cases of 7.

169 adult hamsters (*Mesocricetus auratus*) were utilized in this experiment, 102 were autopsied. The tumor and lymph nodes were selected on the histological test, fixed in Boun's fluid and stained with hematoxylin and eosin.

Reestablishment cell culture of tumoral tissue were made in the following: one animal with subcutaneously grafted tumor (BHK 21/TSC) was sacrificed under ether narcosis. The fragments of well preserved tumor tissue were trypsinized and 4×10^5 of cells were planted in the bottles. Cell culture

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Figs. 1-3. 1, the TBK 21 culture with fusiform and epithelial-shaped cells and in the center a gigantic macrophage in the state of phagocytosis. Below in the right angle, a mitosis. Passage 19, 48 hours old culture. $\times 470$. 2, hamster with enormous BHK 21 tumor on the back. 3, hamster with metastasis in axillary and inguinal lymph nodes. Multiple metastatic foci in the lungs. A metastasis in the left kidney. Autopsy.

of intradermal grafted tumors (BHK 21/TID) was elaborated on the same manner.

Results

A) *Characteristics of the original BHK 21 cells.* The cell layer was composed mostly of atypical spindle-shaped cells, than of polygonal epithelial-shaped cells, connected by the cytoplasmic processes and gigantic epithelial phagocytes, approximately of $25-75\mu$ in diameter. The last cells has uncommon nuclei with non uniform dispersed chromatine, which increased to 7 in number. In the nuclei have observed dark colored round, oval or irregular formed multiple nucleoli. The cells were criss-crossed oriented, indicating high metabolic activity, which could be registered by the rapid acidification of the medium (Fig. 1).

The optimum growth of cells were obtained with dose of $2,5 \times 10^5$ /ml, forming the complete layer within 78 hours apt to trypsinization (Charte 1).

Chart 1. Curve of growth of the BHK 21 cell line, passage 47. CM: change of nutritive medium.

The enumeration of the chromosomes in the metaphase plate revealed the modal value of 43, within the interval of 10-176. The majority of the cells have the chromosomes in the diploid area, next to hypo- and polyploid (Charte 2).

B) *Behaviour of the BHK 21 cells in the hamsters.* On the place of the inoculation of 5×10^8 cells, tumors appeared in all of the conditioned animals with cortisone, but with some difference. Intradermal implanted cells

Chart 2. Chromosome number distribution of the BHK 21 cells passage 19 and 21.

produced the tumors with fast periferal necrosis, but intraperitonely tested animals had tumors with hemorrhagic ascites. Obtained results led to opine that it is a transformed cell line, which were confirmed by the next experiments.

The comparativ study revealed, that cell density for tumor producing was in extent from 1×10^6 — 5×10^2 /ml, growing in 100% of inoculated non

Table 1. Development of tumors in the hamsters after implantation of BHK 21 culture, P-19 and 20 within six months observation

Cell density	Inoculation					
	ID			SC		
	Animals	With tumors inoculated	Latent period in days	Animals	With tumors inoculated	Latent period in days
1×10^6		6/6	7		6/6	9
1×10^5		6/6	7		6/6	10
1×10^4		6/6	7		6/6	12
1×10^3		6/6	7		6/6	15
5×10^2		6/6	23		6/6	29
1×10^2		4/5	34		5/6	32
5×10^1		3/6	40		0/6	—

Figs. 4-7. 4, BHK 21 sarcoma with fusiform, round and globular cells, oriented in the fascicles and swirls, some in mitosis. Histological section. $\times 360$. 5, sarcoma with predominated round, globular cells and with acidophilic, monstrous gigantic cells, irregular vacuolated with bizarre multinucleated nuclei without fixed position, rounded with clear space. $\times 230$. 6, a large metastasis in the lung tissue composed of fusiform and round cells. $\times 90$. 7, metastasis in the intestinal tract. The normal tissue is displaced by the presence of the metastasis. $\times 90$.

conditioned animals for approximately of 7-23 days by the intradermal implantation, and for 9-29 days by the subcutaneous (Table 1). It can be seen from the same table, that ID method had more significant threshold for testing of tumorigen cell density, then SC one, because tumors developed with only 50 of cells in 3/6 grafted animals.

Macroscopically tumor is presented as sarcoma of nodular surface, firm consistency, infiltrating the adjacent tissue, whitish, slightly vasculated and its growth reaching some time gigantic proportions; meanwhile the average weight was of 30-40 g (Fig. 2).

Microscopically tumor is polymorphous sarcoma, composed of slightly basophilic fibroblasts, round and globular cells, some in mitosis and less frequently bizarre, monstrous, gigantic, eosinophilic, multinucleated cells with cytoplasm sometimes irregular vacuolated. The nuclei of last cells did not have a fixed position, displaced to the periphery or in centre of cells. The cells measured approximatively up to 200μ in diameter and rounded with clear space. These forms of giant cells are rarely observed in the metastases.

Architectonically, the tumor did not have a definite model. Sometimes the cells are oriented in bunches or swirls, but more frequently without a definite orientation and with the blood vessels habitually of cavernous type (Figs. 3 and 4). The BHK 21 sarcoma was transplantable in series. They were made 6 passages of tumor by intradermal mode of grafts, and 5 by subcutaneous. The latent period of growth was shorter for the tumor grafted intradermal (4-5 days), than the tumor grafted subcutaneous (5-6 days, Table 2).

Table 2. Intradermic and subcutaneous passage of BHK 21 tumors in the hamsters

Passage	Implantation					
	ID			SC		
	Animals	With tumors inoculated	Latent period in days	Animals	With tumors inoculated	Latent period in days
1		6/6	5		5/6	5
2		6/6	5		6/6	5
3		5/6	4		5/6	6
4		6/6	5		6/6	5
5		6/6	4		7/7	5
6		7/7	4		—	—

The average life of the hamsters with BHK 21 sarcoma was about 60-90 days. The animals died with signs of cachexia and metastasis developed more frequently in the lymph nodes and lungs (Fig. 6), but presented them in variable percentage in all the organs. The disposition of metastases is presented on Table 3 and their number sharply increased 30 days after the tumor transplantation.

Figs. 8-12. 8, tissue culture of subcutaneous implanted tumor (BHK 21/TSC) composed of fine, lengthened fibroblasts with oval or round nuclei, oriented in one direction. In the center a mitosis. Passage 2, 48 hors. $\times 220$. 9, the BHK 21/TSC culture, passage 7 demonstrates signs of degeneration. The cells are round, globular, pycnotic, crowded in irregular groups. Some atypical fibroblasts with extremely long cytoplasmatic prolongations. $\times 370$. 10, culture of intradermal implanted tumor (BHK 21/TID), passage 15. The cells

C. Characteristics of the reestablished tissue cultures of the tumors.

a) The culture of the subcutaneous grafted tumor (BHK 21/TSC) has grown rapidly and abundant at the beginning, forming a layer within 72 h., composed exclusively of fine, long and thin fibroblasts, oriented in one direction, with oval nucleus and chromatine in irregular masses (Fig. 8), having oval or round, dark nucleoli, one till five in number. Suddenly, from the 3rd passage, the growth began to diminish gradually, demonstrating symptoms of degeneration with rounding and clumping of cells. It was noted the change of their morphology, the presence of round, globular, pycnotic cells and rare atypical, monstrous fibroblasts, having long cytoplasmatic prolongation, forming irregular groups of crowded cells, remaining clean spaces between them (Fig. 9), replacing the normal form of fibroblasts. After the 7th passage, the culture had disappeared.

b) The culture of the intradermal grafted tumor (BHK 21/TID) within 72 h also formed a complete layer, but with a little abundance of cells. The initial growth was irregular with spindle and epithelial shaped cells and from 50th passage the culture has adopted a uniformly growth (Figs. 10-12). The monolayers were composed of irregular polygonal, exclusive epithelial-shaped cells, presented round and oval nuclei, two or three in number and chromatin dispersed in the form of irregular balls and bands and with

are polygonal, epithelial formed and with scarce fibroblasts. In the center an enormous monstrous macrophagus with the nucleus acquiring the shape of a sickle at the moment of digesting cells. $\times 280$. 11, the BHK 21/TID culture, passage 28. In the majority the cells are epithelial and some spindle-shaped. In the center a monstrous, gigantic macrophage with irregular, multiform polyglobulated nucleus at the moment of phagocytosis. $\times 220$. 12, the BHK 21/TID cell line, passage 55. The cell monolayer is composed of epithelial-shaped cells. In the center a monstrous, gigantic macrophagus with one enormous nucleus in the final phase of phagocytosis with detritus in the cytoplasm. $\times 240$.

Table 3. Metastases of BHK 21 sarcoma in hamsters at different periods of time

Group	Life with tumors in days	Animal with metastases			Total	Metastases in organs									
		Without	One organ	Various organs		Lymph nodes	Lungs	Kidneys	Heart	Liver	Spleen	Digest. tract	Adrenal glands	Pancreas	Sexual glands
A	0-15	—	7	10	17	16	10	3	—	4	—	—	—	—	—
B	16-30	1	10	21	32	26	25	3	1	2	3	2	1	—	—
C	31-60	2	3	34	39	33	34	23	11	7	6	3	3	1	—
D	61-90	—	—	14	14	13	14	9	6	5	4	1	—	—	1
Total		3	20	79	102	88	83	38	18	18	13	6	4	1	1

more than one dark nucleoli. It was noted too, the presence of extremely bizzare and monstrous macrophagos, exceptionally large, epitheliiforme-shaped, approx.

Figs. 13-15. Three figures of metaphase plates of BHK 21 cells. 13, part of a gigantic cell; unusual forms of chromosomes of BHK 21 cells; passage 19. 14, dicentric forms of chromosomes with large size in hypodiploid number; passage 19. 15, metaphase plate of BHK 21 cell; middle size of chromosomes with dicentric form; passage 19.

75–350 μ and more, with particularly irregular nuclei in form and number, demonstrating a enormous possibility for phagocytosis (Figs. 10–12).

The optimum density of cells for growth were of 3×10^3 /ml, forming a complete layer within 72 h with intensive metabolism, giving cells in abundance (Chart 3).

The line acquired a modal value of 87 chromosomes with diapason of 2–397, demonstrating a high aneuploidy. The majority of cells had chromosomes in the diplo and tetraploid zone, besides hypo- and poliploidy (Chart 4).

Chart 4. Chromosome number distribution of the BHK 21/TID cells passage 46 and 52.

Discussion

Based on the obtained results, it can be concluded, that presented BHK 21 cell lines have all characteristics of oncogenic converted cells: morphological heterology, chromosomal aneuploidy and high metabolic activity. These cells implanted in adult non-conditioned animals, induced infiltrate and metastasized pleomorphous sarcoma. Its possibility of growth in baby mice, rats and chorio-allantoic membrane (Traykovich 1968b), verifies the oncogenic transformation of cells (Traykovich 1968a), produced spontaneously in long-term culture. From mentioned behaviour, the line is similar to the culture of spontaneous tumors (Moore *et al.* 1967, Traykovich and Rivière 1967a).

The new peculiarities acquired of the BHK 21 line under influence of the passage in the susceptible animals, were meaningful for the restored cultures of subcutaneously and intradermal grafted tumoral tissue.

The tissue culture of subcutaneous tumor (BHK 21/TSC) demonstrated in every sense as the normal fibroblastic culture with lengthened and thin spindle-shaped cells oriented in one direction, but without faculties for survival. This phenomenon can have these explanations: a) that the fragments of tumor used for the culture were composed of the mature fibroblasts, because the tumor was a polymorphous sarcoma; b) that the spindle-shaped cells present in the culture originated of conjunctival stroma of tumor tissue; or c) that the tumor in the culture for the unknown reason adapted the particularities of the normal cells by means of differentiation. This phenomenon in the culture of BHK 21/TSC normal tissue is uncommon and significant

by the reversion of the tumor in the normal tissue.

On the other hand, the culture of intradermal grafted tumor also presented its particularity: epithelial structure of cells with very uncommon gigantic cells—monstruous macrophages. For these reasons, the line is clearly distinguished from the original BHK 21 culture (Table 4).

Table 4. Comparative characteristics between BHK 21 and BHK 21/TID cell lines

Characteristics	Denomination of culture	
	BHK 21	BHK 21/TID
Morphology	Majority cells spindle-shaped, epithelial less, and macrophages scarce, approx. diameter $25-75\mu$	Epithelial cells and scarce, atypical, monstrous gigantic macrophages, approx. diameter $75-350\mu$ and more
Modal value and zonal number of chromosomes	43 (hypoploide) 10-176	87 (hypotetraploide) 2-397
Optimum growth cell concentration	$2,5 \times 10^5$	3×10^5
Minimum tumoral cell dose for the hamsters	5×10^2 Appearance of tumors within approx. 23 days by ID graft, and 29 days by SC graft	?
Tumoral type	Polymorphous, metastatic and transplantable sarcoma	?

It is the opinion that the derivatives of BHK 21 cells, the TSC and TID cells have their origin in the original BHK 21 culture and also in the BHK 21 tumors, due to the fact that they contain similar cellular types. By means of the passage of original cells in the susceptible animals were effectuated cellular selection. Sanford *et al.* (1959) wrote about malignant increase of some cellular line of mice by the grafts within animals of the same genotype. Similar results have been reported by Klein *et al.* (1954). Garzčić and *et al.* (1965) also found certain changes in the properties of the L line after implantation in the C3H mice and returned in to vitro conditions.

It was noted that the phase of adaptation have not existed for the BHK 21 tumors in the tissue culture, as in the case of spontaneous tumors of hamsters. This finding is in correlation with Stone's opinion (1962), that long-term cultures suffered the changes of genotype in the sense of tetra- and polyploid. The cells in these forms in the culture tissue are more adaptable, than the diploid cells for in vitro condition, that was the case with BHK 21 cells and theirs tumors.

In the culture of intradermal grafted tumors (TID) was observed the presence of the monstrous, gigantic macrophage with exceptionally uncommon bizzare nuclei (Figs. 10-12). This cellular type can be formed in the following manner due to imperfect karyodiarisis: a) by the synchronous or

asynchronous mitosis of the nuclei ; b) by the endoreduplication of the chromosomes ; or c) by the germination of the nuclei without cytodiareisis, obtaining a diameter of $75-350\mu$ and more (Figs. 10-12). These cells by means of agglomeration, phagocyte the others cells in which the nuclei actively intervenes (Figs. 10-12). It could be that the cannibalism is a manner of these macrophages to supply the indispensable nutritive material, besides the osmosis from the nutritive medium, present in the tissue culture. The preliminaries of these cells were found in the original BHK 21 culture, but having the dimension much more reduced and in tumor tissue (Fig. 5) too. The similar cells were also observed in the human tumoral tissue. Chomette D. *et al.* (1967) of the French school, described similar giant cell type in the bronchopulmonar epithelioma of the human species. One possible conclusion is made, that these monstrous macrophage in the tissue culture of the original BHK 21 line, in the tumors as well as in the BHK 21/TID line are identical cells, but uncommon in the metastatic foci because of their rare frequency, voluminosity and immobility.

It was concluded too, based on the results showed on Table 1, that intradermal testing of cell density on potency for tumor producing has a significant threshold that of subcutaneous mode, and can be explained by richness of capillary network of the dermis, than the subcutaneous tissue.

Summary

It was presented a correlative study in the modification of BHK 21 cells, during its inoculation in hamsters, and after restoration the tissue cultures from tumors.

The original BHK 21 culture was composed of spindle-shaped, epithelial-shaped cells and gigantic macrophage with diameter about $25-75\mu$, having chromosomal aneuploidy with modal value of 43 (hypoploid zone). The 500 BHK 21 cells grafted in hamsters produced in 100% of animals metastasizing polymorphous sarcoma with scarce bizarre macrophage and the tumors appeared after approx. 23 days by intradermal grafts and for approx. 29 days by subcutaneous. The tumor had fast growth, 4-6 days after grafting and the animals lived with tumors about 60-90 days. The intradermal testing of cell density for possible tumor producing has a significant threshold to that of subcutaneous mode.

There was obtained by the restoration of the tissue cultures from the tumors: a) the BHK 21/TSC culture from the subcutaneous grafted tumors, composed of fibroblasts, which disappeared in the 7th passage by degeneration ; and b) the new established BHK 21/TID cell line, originated from the intradermal grafted tumor. After the 50th passage, the TID line has definitely acquired uniformity, having epithelial-shaped cells and gigantic, monstrous macrophage, of diameter $75-350\mu$ and more, aneuploidy and modal value of 87 chromosomes (hypotetraploid zone).

The difference obtained in the type of culture between the original BHK

21 cells and its derivatives: the TSC and TID cultures was the consequence of selective passage of the cells within the susceptible animals.

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