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ESTABLISHMENT OF CELL STRAINS FROM PRIMARY MONKEY KIDNEY CELL CULTURES

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The numerous cell strains available in the different laboratories are partly of human, partly of animal, chiefly mammalian origin. They had been obtained from different healthy, neoplastic or embryonal tissues. In some cases no visible changes had ensued in the course of their establishment, in other cases, however, striking morphological changes had occurred i.e. the cells appeared to have undergone "transformation".

Out of the great variety of cell strains some had been obtained from monkeys. Parker[16], Hull, Cherry and Johnson[11], Westwood, Macpherson and Titmuss [27], Simon [22] and Morzycka [15] reported on the successful continuous propagation of monkey kidney cells. Salk and Ward [20] described the establishment of a cell strain obtained from Cynomolgus heart endothelium.

The present paper reports some systematic studies on the establishment of cell lines from pooled and individual monkey kidneys. Some factors influencing the processes involved are also discussed.

Material and methods

A 0.25 per cent trypsin solution prepared from "Difco-trypsin 1 : 200"[1] in a Ca-, and Mg-free PBS [4] was used. The lactalbumin-hydrolysate solution contained 5 per cent NBC lactalbumin-hydrolysate[2] in distilled water. The composition of the initial medium (IM) was: 2 per cent calf serum, 10 per cent lactalbumin-hydrolysate solution and 88 per cent Hanks' balanced salt solution [9]. From the seventh day of cultivation a propagating medium (PM) containing 5 per cent lactalbumin-hydrolysate solution, 10 per cent calf serum, 40 per cent synthetic mixture 199 [21] and 45 per cent Hanks' balanced salt solution was used.

For the preparation of primary monkey kidney cell cultures, young Rhesus and Cynomolgus monkeys of 2 to 3 kg body weight were used. The animals were anaesthetized by injecting 3 ml of a 10 per cent thialbarbital solution[3] intraperitoneally. The animals were killed by bleeding through the carotids. The kidneys were removed, decapsulated and the minced cortex was trypsinized by the method of Youngner [29] at 30° C, with trypsin changes at five minute intervals. Only test-tube cultures were prepared in every case, using 200.000 cells in one ml of medium per tube. Incubation took place at 36° C in a slanted position. The medium was changed weekly throughout.

[1] Difco Corp., Detroit, Michigan.
[2] Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Chinoin, Budapest, Hungary.

Subcultures were prepared by the method of SWIM and PARKER [23] slightly modified by us. The cells were removed from the glass surface with bent tipped glass tubes and resuspended by sucking and blowing (scraping). The first passage was made at arbitrarily chosen times in the different experiments. Each primary culture was considered as the possible parent of a cell line and was thus separated rigorously. Cultures having survived a minimum of ten passages with satisfactory growth were designated as cell lines in contrast to the cell strains characterized by continuous growth after establishment. This arbitrary distinction is based only upon the growth characteristics of cells. Similar distinctions were, however, made also by other authors [5, 24].

For morphological studies slide preparations were made by the collodion membrane method [19]. The random selection of cultures for staining was always made on the seventh day following the last passage or fluid change. The preparations were stained according to PAPPENHEIM.

Experimental

Establishment of cell lines from pooled monkey kidneys. The first series of experiments was performed in order to detect whether it was possible to

Table 1

Establishment of cell lines from pooled monkey kidneys

Primary cell cultures					Lines established				
Batch number	Date of preparation	Species and number of monkeys	Autopsy	Number of tubes	Number	Date of establishment	Incubation before establishment (in days)	Designations	Incidence %
I.	19. VII. 1959	6 Cynomolgus	negative*	100	1	28. X. 1959	103	I/1.	1.0
II.	28. VIII. 1959	9 Rhesus	2 T.B.C. 7 negative	12	1	27. I. 1960	152	II/1.	8.3
III.	25. IX. 1959	6 Rhesus	negative	24	1	29. XII. 1959	95	III/1.	4.2
IV.	29. IX. 1959	4 Rhesus		30	0				0.0
V.	2. X. 1959	4 Rhesus		33	0				0.0
VI.	7. X. 1959	6 Rhesus	negative*	100	1	19. V. 1960	224	VI/1.	1.0
VII.	9. X. 1959	6 Rhesus	negative*	31	0				0.0
VIII.	13. X. 1959	7 Rhesus	negative*	34	4	29. XII. 1959 29. I. 1960 29. I. 1960 26. II. 1960	77 108 108 135	VIII/1. VIII/2. VIII/3. VIII/4.	11.7
IX.	20. X. 1959	4 Rhesus	pneumonia	30	0				0.0
X.	23. X. 1959	1 Rhesus	dysentery	30	0				0.0

* Previously used for control of Salk vaccine.

obtain cell lines under the experimental conditions used. For this purpose ten different batches of cell cultures were prepared. The number of tubes in each batch was different. The cell suspensions were obtained by trypsinization of the pooled kidneys of several monkeys (see Table 1). The cell cultures were incubated for 77 days with weekly fluid changes. At that time each culture was scraped off after fluid change and the suspended cells were allowed to re-settle in the same tube. In the course of further cultivation, cell growth was examined weekly.

As it may be seen in Table 1 there was only one tube exhibiting satisfactory growth on the seventh day after scraping. This was line No VIII/1. The rest of the tubes showed very poor or no growth one week after scraping. A few large, flat, granulated cells could only be detected in the tubes. On further incubation, however, small groups or islands of cells were found in some of the tubes. These new cells were allowed to multiply until sufficiently large islands had developed. Then they were scraped off again and either allowed to grow in the same tube, or transferred to another. Some of these cultures perished after two or three passages, others gave rise to a cell line. Out of the ten tissue culture batches five did not yield any cell line. Four lines were obtained from one batch, while one line from four batches each.

Establishment of cell lines from individual monkey kidneys. In the second series of experiments 160 tube-cultures were prepared from the kidney cell suspensions of four individual monkeys each (see Table 2).

After 7, 28, 35, 42, 49, 56, 63 and 70 days of incubation 20 tubes each were scraped off from every one of the four different tissue culture batches. Medium change and examination of growth were made in the same way as in the first series of experiments. Cells subcultured on the 7th day were found to have survived 4 to 5 successive passages at 7 day intervals. Attempts to subcultivate primary cells incubated for more than 7 days either failed or succeeded only once, thus later such cultures were never subcultured after scraping but were allowed to re-settle in the original tube. After a lag period of different duration, the development of small islands could be detected in some of the tubes. The morphology of the cultures during both the lag phase and the island formation was essentially the same as in the first series of experiments. Out of the kidneys of the four individual monkeys, two, designated No XI and No XIV, yielded 7 lines each. No lines were obtained from monkey No XII, while monkey XIII gave rise to as many as 16 lines.

Relation of the number of cell lines from individual monkey kidneys to the date of the first passage and to the period between the start of primary cultures and the development of the lines. Table 3 shows that the earlier the first passage had been carried out, the more lines had developed, i.e. the incidence of line development was inversely related to the incubation time of the primary culture. The frequency of establishment appeared to be related also to the

Table 2

*Establishment of cell lines from individual monkey kidneys**

Primary cell cultures					Lines established				
Batch number	Date of preparation	Species and number of monkeys	Autopsy	Number of tubes	Number	Date of establishment	Incubation before establishment (in days)	Designation	Incidence %
						6. IX. 1960	98	XI/1.	
						6. IX. 1960	98	XI/2.	
						6. IX. 1960	98	XI/3.	
XI.	31. V. 1960	1 Rhesus	negative	160	7	20. IX. 1960	112	XI/4.	4.4
						27. IX. 1960	119	XI/5.	
						27. IX. 1960	119	XI/6.	
						25. X. 1960	147	XI/7.	
XII.	3. VI. 1960	1 Rhesus	negative	160	0				0.0
						1. VIII. 1960	56	XIII/1.	
						15. VIII. 1960	70	XIII/2.	
						15. VIII. 1960	70	XIII/3.	
						22. VIII. 1960	77	XIII/4.	
						22. VIII. 1960	77	XIII/5.	
						22. VIII. 1960	77	XIII/6.	
						22. VIII. 1960	77	XIII/7.	
						29. VIII. 1960	84	XIII/8.	
XIII.	7. VI. 1960	1 Rhesus	Granulomatosis	160	16	29. VIII. 1960	84	XIII/9.	10.0
						29. VIII. 1960	84	XIII/10.	
						5. IX. 1960	91	XIII/11.	
						5. IX. 1960	91	XIII/12.	
						5. IX. 1960	91	XIII/13.	
						11. X. 1960	126	XIII/14.	
						23. XI. 1960	168	XIII/15.	
						12. I. 1961	219	XIII/16.	
						2. IX. 1960	84	XIV/1.	
						9. IX. 1960	91	XIV/2.	
						9. IX. 1960	91	XIV/3.	
XIV.	10. VI. 1960	1 Rhesus	negative	160	7	9. IX. 1960	91	XIV/4.	4.4
						16. IX. 1960	98	XIV/5.	
						30. IX. 1960	112	XIV/6.	
						7. X. 1960	119	XIV/7.	

* Previously used for control of Salk vaccine.

total period of incubation. The earliest establishment took place between the 43—56th days of incubation. From this time on the frequency of establishment increased and reached a maximum between the 85—98th days. Later the development of cell lines became gradually less frequent. These data have shown the influence of the time factor on the development of cell lines. In case of monkey No XIII, the incidence of cell line establishment reached its maximum about a fortnight earlier than in other cases. This observation points to the possible existence of individual differences of the monkeys.

Table 3

*Relation of the number of cell lines from individual monkey kidneys to the date of the first passage and to the period between the start of primary cultures and the development of the lines**

First scraping (day of incubation)	Incubation, days								Total
	1–42	43–56	57–70	71–84	85–98	99–112	113–126	above 126	
7		**1**		**5 6 7**	*1* **11 12** ② ④ **	*4*	*5 6* **14**		13
28			**3**	**8 9** ①	*2*		⑦		6
35			**2**					*7* **15**	3
42					③ ⑤	⑥			3
49				**4 10**					2
56					*3*				1
63									0
70					**13**			**16**	2
Total		1	2	8	10	2	4	3	30

* *1—7* Lines from monkey No XI.
1—16 Lines from monkey No XIII.
①—⑦ Lines from monkey No XIV.
The figures designate lines.
** Critical points of time (for their significance, see the Discussion).

History of cell lines. Fig. 1 shows the history of the lines developed from pooled monkey kidneys.

Lines No VI/1, No VIII/2, No VIII/3 and No VIII/4 after varying periods of satisfactory growth exhibited a gradual decrease of the multiplication rate, resulting finally in the stop of growth. Nevertheless, all these cultures were incubated for further six months with weekly fluid changes. After this period, as none of the residual cells were found to start growing, they were discarded. With line No II/1, too, multiplication appeared to have stopped. After 14 weeks of additional incubation, however, a new population of cells differing from the original one began to develop. These new cells grew readily and yielded strain No II/1/a. Up to now this strain has been carried through 120 passages. From lines No I/1 and No VIII/1 passages in two-litre Roux flasks were also prepared to obtain large quantities of cells. After some transfers, islands of cells somewhat similar to those of strain No II/1/a appeared in these cultures. The new cells replaced successively the old ones and finally the cultures consisted of these modified cells only (strains No I/1/a and No VIII/1/a). At present, strains No I/1/a and No VIII/1/a are in their 100th and 120th passages, respectively. No similar changes were observed in the tube cultures of the same cells

maintained parallel to the cultures in Roux flasks. The former, after having been carried through several passages, died out successively on further incubation of six months. Cell line No III/1 could be propagated without difficulty and showed no changes in morphology, but the cells multiplied at a slow rate. This cell strain has now been carried through 80 passages.

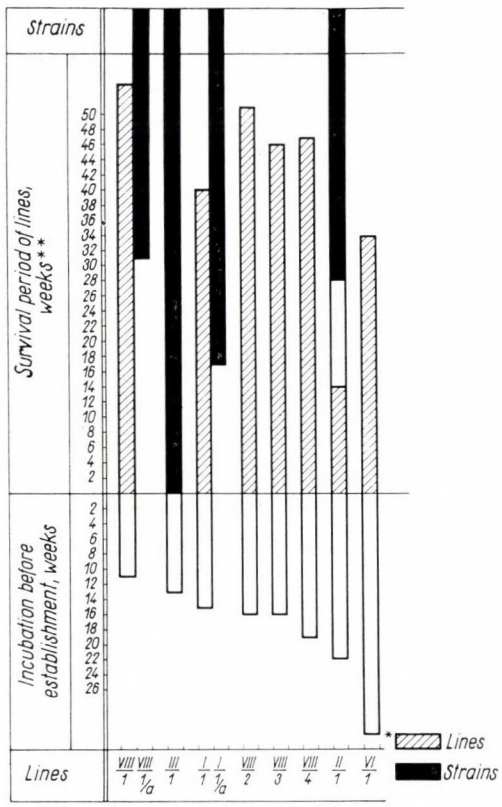


Fig. 1. History of lines from pooled monkey kidneys
** Subcultures were made at one or two week intervals

Fig. 2 shows the history of the lines obtained from individual monkey kidneys. The survival period of cultures varied from monkey to monkey. Lines developed from monkeys No XI and No XIV lived generally longer than those from monkey No XIII. There were differences in the survival period of lines obtained from the same monkey. The survival of the individual lines seemed to be related to the time of their establishment and to the date of the first scraping. The later was made the first passage and had the line developed, the shorter was the period of survival. The rate of multiplication of line No XI/1 from monkey No XI slowed down much earlier than that of the other lines

from the same batch. After a period of very slow growth, however, the line improved and yielded strain No XI/1/a. Line No XIII/12 from monkey No XIII has been propagated easily up to the present time, thus it appeared to have yielded a cell strain with characteristics essentially similar to the parent line. In case of line No XIII/11 from monkey No XIII there was no difficulty in

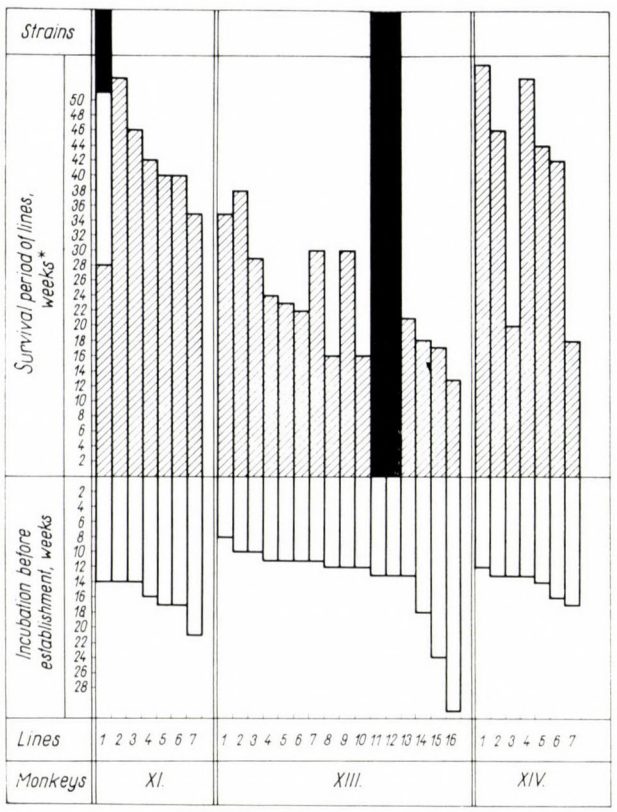
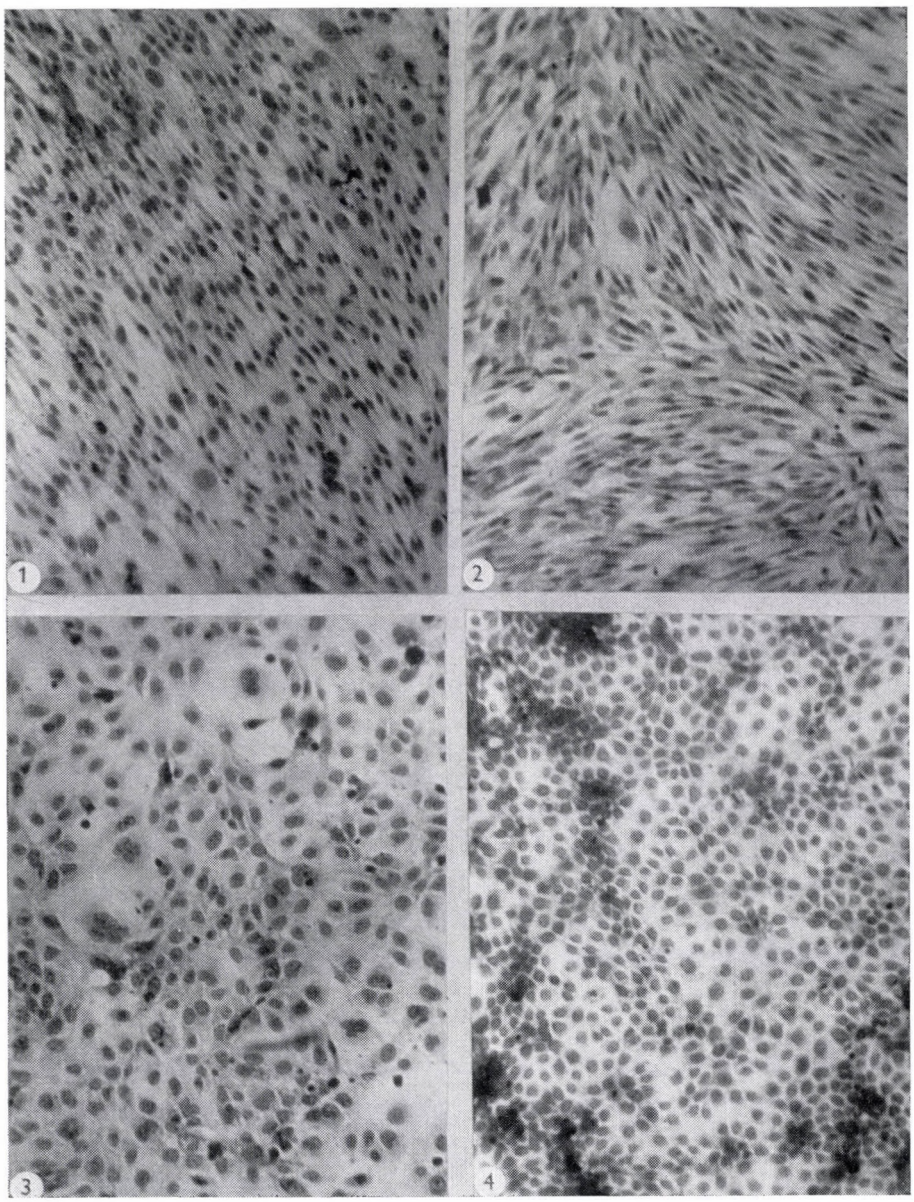


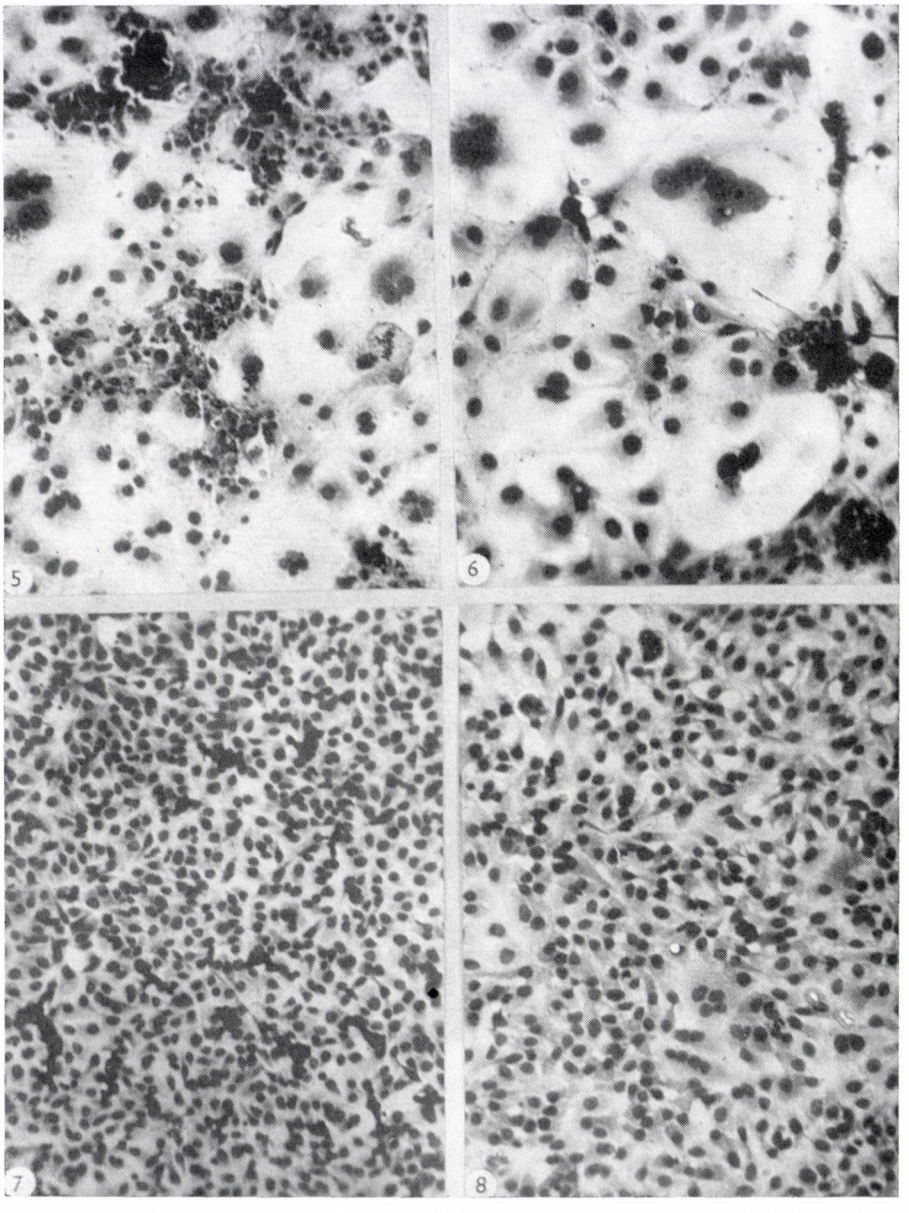
Fig. 2. History of lines from individual monkey kidneys
* Subcultures were made at one or two week intervals

propagation. There was, however, a period when part of the cultures exhibited poor multiplication as compared to the others. This labile period lasted for a few weeks. It seems to be of interest that all these three strains were obtained from primary cultures subcultured first on the seventh day of incubation and from lines established between the 85—98th days (see Table 3). Up to now these strains have been carried through 50, 80 and 70 passages, respectively.

Morphology. Part of the lines obtained were fibroblast-like, elongated, spindle-like in form, and characterized by an oriented bundle-like growth. Lines No III/1, No VIII/1, No VIII/2, No VIII/3, No VIII/4 and those from



1. Line No VIII/1. 2. Line No XIV/2. 3. Strain No XIII/12. 4. Line No XI/7.
Photos were taken by a Row. microphot B. apparatus, ocular 5 and objective 20, magnification, ×60.



5. Line No I/1. 6. Line No II/1. 7. Strain No I/1/a. 8. Strain No II/1/a.

Photos were taken by a Row. microphot B. apparatus, ocular 5 and objective 20, magnification, ×60.

monkey No XIV belonged to this type (see Photos 1 and 2). All the other lines and strains (except for strain No III/1) were epitheloid in character. The polygonal cells formed crazy-pavement-like cell sheets, without any orientation (see Photos 3 and 4). Within these two main types of culture the individual cells of the lines exhibited a great degree of polymorphism. The size of cells showed great variety. Many large, flat cells and cells exhibiting atypical mitoses or several nuclei were frequent. After establishment of the strains the polymorphism was greatly reduced (see Photos 5, 6, 7 and 8). Detailed data on the related morphological problems will be presented elsewhere.

Discussion

Most authors [1, 7, 8, 13, 14, 17, 24, 27] agree in that after a short period of satisfactory primary growth a lag phase of different duration has to pass before the cell strain would develop. In some cases, however, the established cell population appeared without a remarkable decrease in the rate of multiplication of the culture as a whole [2, 3, 10, 12, 20, 26, 27]. This type of cell strain establishment is characterized by the development of small initial islets of new cells in the sheet of the original ones. As these new cells grow usually better on further cultivation, they successively overgrow the original cell population. The new cells are usually regarded as "transformed" ones. Sometimes neither a decrease of growth nor the appearance of a new cell type occurred [6, 18, 25, 27, 28] and the strain was established without any visible morphological changes. Such strains are generally considered as "non-transformed" ones. Nevertheless, in the latter case, too, there are differences between the new and the original cells, i.e. strains grow usually better than primary cultures and it is usual to find an increased chromosome number.

According to the former distinction, the lines and strains presented should have been regarded as "transformed" as a lag period during their development occurred in every case and the new cells were morphologically more or less unlike the original cells. The development of the new population seemed, however, to result not of a sudden change, but of a slow process. This could readily be followed under the microscope. During the lag phase a few cells began to multiply, forming small islets. The fates of these islets were different. Part of them died out after they had reached a certain size. Some of them had been carried through two to three passages before they perished. In other cases, however, they gave rise to cell lines.

In case of lines obtained from individual monkey kidneys, the date of first passage and the behaviour of cells seemed to be related, i.e. the earlier was the first passage performed, the more lines developed. Thus the actual rate of multiplication appears to have a certain role in the early establishment

of cell lines. The period of survival of the lines seemed to be related also to the date of their establishment and to that of the first passage. In our experiments there seemed to be two critical points of time during the establishment of cell strains. The first was the passage on the 7th day and the second the appearance of a line between the 85—98th days. Later passage of the primary cultures and the appearance of a line out of the above limits of time led in no case to the establishment of a cell strain. This phenomenon could not be ascribed to changes in the nutrient medium, as media were always prepared from identical ingredients by the same methods throughout the experiments. Considering the data obtained, the establishment of cell strains under the experimental conditions used might be the result of a two-phase process influenced by the time factor. During the first phase labile cell lines had developed. These lines either died out after a certain period or had gone into the second phase of establishment. The latter took place only in lines developed under the above mentioned special conditions of timing.

Establishment of strains occurred only in lines of epitheloid character. The lines derived from monkey No XIV did not yield any strains even in the optimal period. These cells, however, were fibroblast-like and survived for about a year. The poor adaptability of human fibroblasts to continuous growth in tissue culture has been described [18, 24]. The same appears to be true for monkey fibroblasts. As an explanation of the lack of establishment of cell strains from cultures characterized by the prevalence of fibroblast-like cells, it may be supposed that the few epithelial cells present at the beginning were completely overgrown and lost during the passages. Of the cultures prepared from monkey No XII not a single line could be obtained. Under the experimental conditions used these cells were apparently unable to multiply *in vitro* for a longer period. These data show that the behaviour of cells during cultivation may depend on certain individual factors.

The cells giving later rise to a line are probably present in the primary cultures. Their number is, however, very small so that they are overgrown by the other cells. Nevertheless, they survive and begin to grow after the death of the previously prevalent cells. The fact that in case of monkey No XIII the lines developed earlier and at a more than twice as high incidence, than in the other two cases, seems to support that assumption, since primary cultures had begun to degenerate during the second week of incubation and in the third to fourth week there were only a few cells detectable in the cultures. If the above supposition were correct, one should have expected an early establishment of strains. This was, however, not the case in our experiments, as we have found that the establishment of cell strains depended in this case, too, on the two critical points of time mentioned above. We must also take into consideration the fact that monkey No XIII proved to have granulomatosis at autopsy. This may have caused the special culture characteristics of the

cells derived from this monkey, but does not explain the critical points of time during the development of strains.

The lines obtained from the pooled monkey kidneys contained cells from different animals mixed at random. It was demonstrated above that cells from different individual monkey kidneys showed different characteristics in tissue culture. It is reasonable to suppose that a mixture of cells of different characteristics (such as those from pooled kidneys) might exhibit a particular behaviour. Observations on line No VIII/1 have shown that this may happen, viz. from a culture of apparently fibroblast-like character islands of epitheloid cells grew out later.

More detailed data on the morphological and genetical observations made during this work will be presented elsewhere.

Summary

1. The establishment of 8 cell lines from 10 different batches of cultures prepared from cell suspensions of pooled monkey kidneys and that of 30 lines from 4 individual monkey kidneys are described.

2. Among the 38 cell lines 7 yielded permanent cell strains.

3. The conditions favourable for the establishment of lines and strains have been discussed from the point of view of the date of first passage and of the incubation period. The establishment of strains seemed to be the result of certain two-phase process influenced by the time factor.

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ISOLIERUNG VON ZELLSTÄMMEN AUS PRIMÄREN AFFENNIEREN-ZELLKULTUREN

P. RUZICSKA

1. Von den aus 10 verschiedenen, gemischten Affennieren-Zellsuspensionen erhaltenen Kulturen wurden 8 Zellinien, und von den aus 4 Affen individuell erhaltenen Kulturen 30 Zellinien isoliert.

2. Von den 38 Zellinien wurden in 7 Fällen permanente Stämme erhalten.

3. Es wurden die optimalen Bedingungen für die Entstehung von Zellinien und -stämmen in bezug auf den Zeitpunkt der ersten Passage und die Inkubationsdauer untersucht. Die Herausbildung der Zellstämme schien ein Ergebnis eines zweiphasigen Prozesses zu sein, wobei dem Zeitfaktor eine Rolle zufiel.

ОБРАЗОВАНИЕ КЛЕТОЧНЫХ ШТАММОВ ИЗ ПЕРВИЧНЫХ КЛЕТОЧНЫХ КУЛЬТУР ПОЧЕК ОБЕЗЬЯН

П. РУЗИЧКА

1. Из групп культур изготовленных из клеточных суспензий, полученных от почек различных обезьян, дается описание образования 8 клеточных линий, а из культур, полученных из почки отдельных обезьян — образование 30 клеточных линий.

2. Из 38 клеточных линий в 7 случаях получались перманентные клеточные штаммы.

3. Исследовались оптимальные условия образования клеточных линий и штаммов, с учетом срока первого пассирования и продолжительности инкубации. По собранным данным кажется вероятным, что образование клеточных штаммов было результатом двухфазного процесса, на который фактор времени оказал действие.

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