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Attempts at Synchronization of L-Cells by Thermal Shocks

Próby synchronizacji komórek L za pomocą szoków termicznych

Попытка синхронизации клеток L при помощи термических шоков

INTRODUCTION

A number of methods have been applied to synchronize mammalian cells in culture. Most of them were based on the selective inhibition of DNA synthesis by means of amethopterin (22), fluorodeoxyuridine (9, 18), deoxyadenosine (4, 3, 28), deoxyguanosine (4, 28), or by means of an thymidine excess (3, 28). Synchronous induction of cell division was also performed by the application of mitotic inhibitors such as colcemid (21) or vinblastin sulphate (6). Synchronization based on the inhibition of DNA synthesis and mitotic divisions however, may cause changes in the metabolism of cells under treatment. The centrifugation of the cells in sucrose gradient was used as one of the first methods of obtaining a synchronous population (23).

The observation that mitotic cells adhere much less firmly to glass or plastic surfaces than do interphase cells suggested the use of mechanical separation of such cells to obtain synchronous cell populations (26). To increase the yield of this method (26) removal of calcium ions from the growth medium (20) or short cooling of the culture and addition of trypsin (24) were applied to some lines.

Thermal shocks for synchronization of division of HeLa cells were first proposed by Newton and Wildy (15). Miyamoto, Rasmussen and Zeuthen (11) introduced this method for synchronization of mitoses in L-cells.

Our attemps to synchronize L-cells by thermal shocks are discussed in this paper.

MATERIAL AND METHODS

Medium — The culture medium used was that introduced by Parker supplemented with 10% of calf serum (inactivated for 30 minutes at 56°C), 100 units of penicillin and 60 μg of streptomycin per 1 ml. The Parker medium and the calf serum were purchased from the Producers of Sera and Vaccines in Lublin.

Harvesting of cells — The cells from 2 or 3 day-old cultures were harvested after treatment with 0.25% trypsin solution at 37°C for 3 minutes. The cell suspensions were then thoroughly mixed by means of a Moore pipette.

Tests — For determination of the viability of the cells a 0.02% solution of erythrosine B (16) was used and the cells were counted in a Burker chamber, whereas for determination of mitotic index the cells were fixed with methanol and stained by the method of May-Grünwald Giemsa modified by Jacobson and Webb (5).

EXPERIMENTS AND RESULTS

For thermal shock experiments the cell suspension with the density of 10^5 cells per ml of the medium was placed in Leighton flasks containing a cover glass each. After cultivation for a definite period of time without a change of the medium, the cover glasses were removed and the cells were fixed and stained as denoted in Material and Methods.

Two types of experiments were performed. In the first type of experiments the cells were subjected once to a cold shock at 4°C for definite

Table 1. Mitotic indices of L-cells as affected by cooling at 4°C for 1 hour (mean values of MI of 3 series each of 5 cultures)

Hours after inoculation		25	26	28	31	43	49
Hours of reincubation at 37°C		0	1	3	6	18	24
MI	Control ‰ ±S.E.	62.1 ±3.17	63.4 ±2.27	55.7 ±1.76	51.0 ±1.67	33.3 ±1.15	25.0 ±1.85
	Experimental ‰ ± S.E.	44.1 ±2.38	49.5 ±2.76	57.3 ±3.71	64.5 ±3.0	33.1 ±1.16	30.1 ±1.35
	Experimental % of control	71.0	78.1	103.0	126.5	99.5	124.0

For technical details see Material and Methods.

Table 2. Distribution of mitotic phases in L-cell population after 1 hour at 4°C

25 hrs after inoculation	Mitotic indices ‰ ±S.E.	Phases of mitosis %			
		prophase	metaphase	anaphase	telophase
Control	62.1 ±3.17	34.6	38.8	2.9	23.7
Experimental	44.1 ±2.38	10.1	47.8	2.0	40.1

Table 3. Mitotic indices of L-cells as affected by cooling at 4°C for 3 hours (mean values of MI of 3 series each of 5 cultures)

Hours after inoculation		27	28	30	33	45	51
Hours of reincubation at 37°C		0	1	3	6	18	24
MI	Control ‰ ±S.E.	58.5 ±2.28	55.7 ±1.76	57.3 ±2.80	40.4 ±1.30	29.7 ±1.65	26.1 ±1.37
	Experimental ‰ ±S.E.	45.5 ±2.30	50.1 ±2.49	76.7 ±2.80	57.0 ±3.02	28.7 ±1.97	25.1 ±2.01
	Experimental % of control	77.8	89.9	133.9	141.0	96.6	96.1

Table 4. Distribution of mitotic phases in L-cell population after 3 hours at 4°C and subsequent reincubation for 3 and 6 hours at 37°C

	Mitotic indices ‰ ±S.E.	Phases of mitosis %			
		prophase	metaphase	anaphase	telophase
Control*	58.5 ±2.28	28.9	39.8	2.9	28.4
Experimental	45.5 ±2.30	14.9	45.1	0.4	39.5
Control**	57.3 ±2.80	25.2	51.1	2.1	21.5
Experimental	76.7 ±2.80	26.7	40.8	2.2	30.3
Control***	40.4 ±1.30	30.3	30.7	3.0	35.9
Experimental	57.0 ±3.02	21.9	40.6	3.3	34.2

* 27 hrs after inoculation, ** 30 hrs after inoculation, *** 33 hrs after inoculation. inoculation.

periods of time. For this purpose 24 hour cultures growing in Leighton flasks at 37°C were chilled at 4°C for 1, 3 or 6 hours and then fixed, whereas the parallel cultures were reincubated at 37°C for 1, 3, 6, 18 or 24 hours before fixation and staining.

In the second type of experiments 24 hr-old cultures growing at 37°C were heated at 41.5°C for 1 hr, then fixed or reincubated at 37°C similarly as in the experiments of the first type.

L-cells cultivated at 37°C for the same periods of time were used as the control. The experiments were repeated 3 times with 5 parallel cultures for each variant. The mitotic indices were calculated after counting the number of mitosis per 1000 cells in 15 parallel cultures.

When tested after 1, 3 or 6 hours at 4°C the L-cells in culture showed a transient decrease in values of mitotic indices, and in percentages of prophases (Tables 1—6). The largest decrease was recorded after 6 hours of cooling. After reincubation of the cells at 37°C a gradual restoration of the mitotic activity was observed. The maximum increase of mitotic index occurred in L-cells cooled for 3 hours and reincubated for 3 and 6 hours at 37°C (Table 4).

The mitotic indices in these cases did not exceed 8%, even though in comparision with the control series these indices increased by 35% and 40% respectively.

Table 5. Mitotic indices of L-cells as affected by cooling at 4°C for 6 hours (mean values of MI of 3 series of 5 cultures)

Hours after inoculation		30	31	33	36	48	54
Hours after reincubation at 37°C		0	1	3	6	18	24
MI	Control ‰ ±S.E.	57.3 ±2.80	51.0 ±1.67	40.4 ±1.30	37.4 ±2.40	26.1 ±1.35	21.5 ±1.53
	Experimental ‰ ±S.E.	30.5 ±2.65	39.5 ±2.60	50.1 ±2.63	43.6 ±1.85	30.5 ±1.83	26.9 ±2.48
	Experimental % of control	53.2	77.5	124.0	116.5	116.8	125.1

Table 6. Distribution of mitotic phases in L-cell population after 6 hours at 4°C

30 hrs after inoculation	Mitotic indices ‰ ±S.E.	Phases of mitosis %			
		prophase	metaphase	anaphase	telophase
Control	57.3 ±2.80	25.2	51.1	2.1	21.5
Experimental	30.5 ±2.65	7.2	59.7	1.5	31.7

Table 7. Mitotic indices of L-cells as affected by heating at 41.5°C for 1 hour (mean values of MI of 3 series of 5 cultures)

Hour after inoculation		25	26	28	31	43	49
Hour of reincubation		0	1	3	6	18	24
MI	Control ‰ ±S.E.	64.6 ±1.24	62.5 ±1.88	63.4 ±2.67	38.8 ±2.14	32.3 ±1.46	22.2 ±1.32
	Experimental ‰ ±S.E.	37.9 ±2.41	63.5 ±3.18	57.9 ±3.28	35.8 ±2.05	28.1 ±1.00	25.6 ±2.07
	Experimental % of control	58.6	101.6	91.3	92.3	87.0	115.3

Table 8. Distribution of mitotic phases in L-cell population after 1 hour at 41.5°C and subsequent reincubation at 37°C for 1 hour

	Mitotic indices ‰ ±S.E.	Phases of mitosis %			
		prophase	metaphase	anaphase	telophase
Control*	64.6 ±1.24	22.7	37.8	3.8	35.7
Experimental	37.9 ±2.41	21.9	38.3	2.5	37.3
Control**	62.5 ±1.88	22.6	30.7	4.2	42.3
Experimental	63.5 ±3.18	28.6	34.3	3.6	33.5

* 25 hrs after inoculation.
** 26 hrs after inoculation.

The transfer of 24 hour-old cultures of L-cell to 41.5°C for 1 hour caused a decrease in mitotic activity by almost 40% (Tables 7, 8), without any change in the percentage distribution of the particular phases of mitosis.

The cells reincubated at 37°C after 1 hour exhibited the same rate of replication as the control cells; this was also the case with all phases of mitoses (Table 8).

When our cultures entered the stationary phase after about 40 hr post inoculation a gradual decrease in mitotic indices both in the control and the experimental series took place.

DISCUSSION

Effects of cooling or heating on mammalian cells cultured in vitro depend not only on the time of exposure to the abnormal temperature and its height, but also on the type of cells, their age and conditions of culture (1, 2, 8, 11, 13, 14, 27).

A decreased viability of different mammalian cells has often been observed after a prolonged incubation at lowered temperature (14, 25). In the cold-adapted LCH-cells the increased number of polyploid cells has also been reported (1). The inhibition of DNA synthesis in HeLa cells and prologation of the generation time have been found in Chinese Hamster cells (13, 15).

Clones with a short generation time of the neoplastic cells H.Ep-2 from the human skin cooled for 1 hour at 4°C gave, after 11 hours of reincubation, a population in which 80% of the cells were dividing, whereas clones with longer generation time were not synchronized by cooling (19).

Newton and Wildy (15) who cooled HeLa cells at 4°C for 1 hour to obtain a parasynchronous population observed a decrease in mitotic index just after coooling and a burst of mitoses after reincubation of cells at 37°C for 13—20 hours.

In our experiments directly after cooling L-cells at 4°C for 1 hour a decrease by about 20% of mitotic indices and by 30% of the prophase percentage was observed but no burst of mitoses occurred after reincubation at 37°C. The maximum rate of multiplication, expressed as the mitotic index, occurred after 3 hours of reicubation at 37°C, whereas after 18 hours of reincubation the mitotic indices declined. The prolongation of the time of cooling to 3 or 6 hours caused no synchronization of L-cells either, similarly as it was observed with L-cells investigated by Littlefield (9). When experimenting with FL amnion cells in culture Miura and Utakoji (10) failed to repeat the experiments of Newton and Wildy. Moreover, Miura and Utakoji (10) suggested that HeLa cells in the experiments of Newton and Wildy (15) were, presumably, not in the logarithmic phase of growth at the moment of cooling.

According to Miyamoto et al. cooling to below 10°C for 1 hour only

slinghtly affectes the L-cells and does not lead to mitotic synchronization. On the other hand, heating to 41—42°C for 1 hour caused an actual retardation of mitoses and prolongation of the generation time of L-cells which resulted in a partial synchronization of the examined cell populations (11). After 1 hour thermal shock the transient inhibition of DNA, RNA and protein synthesis (17) and blocking of mitosis (11) were also observed in L-cells.

In our experiments after 1 hour incubation at 41.5°C and 1 hr reincubation at 37°C, 60 cells per 1000 underwent division similarly as in the control cultures. Afterwards, however, the mitotic indices decreased both in the control and in the experimental series. Thus, the application of an increased temperature did not cause even a transient increase in mitotic indices in L-cells population.

The application of the time-lapse photographic method permitted Miyamoto et al. (12) to trace the effect of thermic shocks on L-cell division in a small number of daughter cells originating from one mother L-cell (11). In contrast, in our experiments the L-cells grew in mass populations, and in order to estimate the mitotic index we examined 15,000 cells for each result, which allowed us to evaluate our data statistically. It is also probable that a repeated application of thermal shocks to cells of a definite age and an adequate time of reincubation would produce satisfactory synchronization of L-cells in mass cultures similarly as in *Schizosaccharomyces* or *Tetrahymena* (7).

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STRESZCZENIE

Próbowano uzyskać synchronizację podziałów komórek L przez krótkotrwałe zmiany termicznych warunków hodowli. W tym celu 24-godzinne hodowle komórek L przetrzymywano w temp. 4°C w czasie 1, 3, 6 godz. lub w temp. 41,5°C w ciągu 1 godz., a następnie reinkubowano w temp. 37°C przez 24 godz. Równoległe hodowle, inkubowane w temp. 37°C w odpowiednim czasie, stanowiły kontrolę.

Przeniesienie hodowli komórek L do temp. 4°C powodowało spadek indeksu mitotycznego, a w szczególności zmniejszenie procentu komórek w stadium profazy przy jednoczesnym wzroście metafaz i telofaz. Reinkubacja w temp. 37°C znosiła ten efekt. Jedynie 3-godzinne chłodzenie hodowli dawało w trzeciej godzinie reinkubacji podwyższenie indeksu mitotycznego, lecz tylko do 76,7‰.

Jednogodzinny szok termiczny (41,5°C) powoduje obniżenie indeksu mitotycznego, ale procentowy stosunek faz podziałów mitotycznych pozostaje w przybliżeniu taki sam jak w serii kontrolnej. Już jednak jednogodzinna reinkubacja hodowli w 37°C daje w następstwie populację komórek L o wartościach indeksu mitotycznego wyższych niż w serii kontrolnej.

Wyniki powyższe wskazują, że zastosowanie pojedynczych szoków termicznych nie jest wystarczającą metodą do uzyskania synchronizacji populacji komórek L. w hodowli *in vitro*.

РЕЗЮМЕ

Предпринимались попытки получения синхронизации деления клетки путем непродолжительных изменений термических условий культуры. С этой целью 24-часовые культуры клеток L в течение 1, 3, 6 часов держались в темп. 4°C или же в течение 1 часа в темп. 41,5°C, а потом в течение 24-х часов реинкубировались в темп. 37°C. Культуры, инкубированные в течение соответствующего времени в темп. 37°C, являлись контрольными.

Перемещение культуры клеток L в темп. 4°C вызывало понижение митотического индекса, особенно уменьшение процента клеток в стадии профазы при одновременном росте метафаз и телофаз. Реинкубация в 37°C этот эффект перенесла. Только ежедневное 3-х часовое охлаждение культуры давало в 3-ем часу инкубации повышение митотического индекса, но только до 76,7‰.

Одночасовой термический шок 41,5°C вызывает понижение митотического индекса, но процентное отношение фаз митотических делений остается приблизительно таким же, как и в контрольной серии. Но уже одночасовая реинкубация культуры в 37°C вызывает популяцию клеток L с более высокими значениями митотического индекса, чем в контрольной серии.

Эти результаты свидетельствуют о том, что применение единичных термических шоков для получения синхронизационной популяции клеток L в культуре *in vitro* не является достаточным.