KINETICS OF CELL PROLIFERATION EVALUATED BY DIFFERENTIAL STAINING OF SISTER CHROMATIDS WITH TWO DIFFERENT POINTS OF CELL FIXATION

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The method of differential staining of sister chromatids enables not only sister chromatid exchanges, but also the proliferative activity of the cells, to be evaluated. Many research workers nowadays, when assessing the ratio of cells in different mitotic divisions, use different parameters to express the rate of cell proliferation [1, 2, 8, 10] or they simply analyze this ratio using only one point of cell fixation [4, 13]. However, with this approach it is impossible to differentiate two different processes – emergence of the cells from the G_0 stage and the actual movement of the cells through the cycle. It has been pointed out in a number of publications that the average duration of the cell cycle for human lymphocytes, even in the same cell culture, can vary greatly [5], and it also depends essentially on the composition of the culture medium and the conditions of cell culture [12, 14].

In this paper a simple method of evaluating the kinetics of cell proliferation using two cell fixation points is suggested, and it is pointed out that different conditions of culture affect only the process of emergence of cells from the G_0 stage, whereas the cell population doubling time remains on average constant.

EXPERIMENTAL METHOD

Proliferative activity of cells was studied in cultures of peripheral blood lymphocytes from 23 healthy blood donors aged from 10 to 19 years. There were two series of experiments. In series I blood samples from three donors were cultured in a mixture containing Eagle's medium, 20% bovine serum, and 0.015 ml of PHA (from Difco, USA). 5-Bromodeoxyuridine was added in a final concentration of 10 μg/ml to the cultures at the beginning of the experiment. The total culture mixture was poured onto individual samples, which were cultured in darkness and fixed every 6 h between 54 and 96 h. In the experiments of series II blood samples from 20 donors were cultured under identical conditions with the components of the culture mixture in the same proportions as in series I, but the Eagle's medium was replaced by medium 199, and cells were fixed at 2 points – after 72 and 90 h of culture. To distinguish the 1st, 2nd, 3rd, and 4th mitoses, chromosome preparations were stained by a modified method of sister chromatid differential staining [3]. One hundred cells were counted at each experimental point. Proliferative activity of the lymphocytes was evaluated by two parameters: the proliferation index (PI) [10] and the mean number of cell divisions (N) [2], by the equations:

$$\mathrm{PI} = \frac{\sum_{i=1}^{n} (i \cdot A_i)}{\sum_{i=1}^{n} A_i}, \quad \mathrm{N} = \left(\sum_{i=1}^{n} \frac{A_i}{2^{i-1}} \cdot i \right) \Big/ \left(\sum_{i=1}^{n} \frac{A_i}{2^{i-1}} \right),$$

where i is the mitosis No. and A_i denotes the number of cells of the i-th mitosis.

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TABLE 1. Ratio between Mitoses in Blood Lymphocytes from Three Donors and Parameters of Proliferative Activity with Different Cell Fixation Times (M ± m)

Fixation time, h	Fraction of mitoses, %				Index of proliferative activity of cells	
	1-st	2-nd	3-rd	4-th	PI	*N*
54	87,7±1,9	12,3±1,9	0	0	1,12±0,02	1,07±0,01
60	77,7±5,2	15,7±3,0	6,7±2,3	0	1,29±0,07	1,13±0,03
66	50,3±6,8	43,7±6,4	6,0±1,0	0	1,56±0,07	1,34±0,06
72	33,3±8,2	45,7±1,9	19,0±5,5	2,0±1,1	1,92±0,18	1,54±0,12
78	22,7±7,6	39,3±3,8	34,0±9,1	4,0±2,3	2,19±0,21	1,74±0,16
84	19,7±7,2	35,3±7,7	35,7±12,4	9,3±5,5	2,35±0,25	1,82±0,18
90	19,3±5,6	21,3±7,5	44,3±8,5	15,0±7,9	2,55±0,25	1,90±0,17
96	11,3±1,8	22,0±4,6	48,0±3,5	18,7±8,6	2,74±0,15	2,15±0,10

TABLE 2. Ratio between Mitoses in Blood Lymphocytes from 20 Donors and Parameters of Proliferative Activity of Cells at Two Different Fixation Points (M ± m)

Fixation time, h	Fraction of mitoses, %				Index of proliferative activity of cells	
	1 st	2-nd	3-rd	4-th	PI	*N*
72	49,8±2,5	49,2±2,5	1,0±0,3	0	1,51±0,03	1,34±0,02
90	19,1±0,9	45,7±1,8	25,3±1,6	9,9±1,1	2,26±0,03	1,79±0,02

TABLE 3. Estimation of Kinetics of Lymphocyte Populations Based on Parameters PI and N in Two Series of Experiments

Parameter of kinetics of lymphocyte population, h	Series I		Series II	
	PI	*N*	PI	*N*
Mean doubling time of cell population	24,9	38,6	24,0	40,0
Time of emergence of first cells from G_0 stage	26,2	13,7	35,7	18,4

EXPERIMENTAL RESULTS

The results of the experiments of series I and II are summarized in Tables 1 and 2, respectively. Regression analysis of the data in Table 1 showed that both parameters of proliferative activity of the lymphocytes are linear functions of the cell fixation time ($r^2 = 0.99$; $P < 0.001$). The linear regression equations are in the form: $PI = -1.0529 + 0.0402 \cdot t$ and $N = -0.3557 + 0.0259 \cdot t$ where t denotes the fixation time of the cells (in h). The mean doubling time of the cell population (from the tangent of the angle of slope of the regression line) and the time of emergence of the fastest lymphocytes form the G_0 stage (from the ratio between the regression coefficients) can be determined [2] from the coefficients of the regression equations.

It will be clear from Table 3 that the parameters of proliferative activity of the cells tested gave essentially different estimates of the kinetics of cell proliferation. For instance, when the parameter N was used, the doubling time of the cell population (1/0.0259 = 38.6 h) was significantly longer, whereas the time of entry of the fastest lymphocytes into the cell cycle, calculated as $-(-0.3557)/0.0259 = 13.7$ h, was shorter than the value of these parameters estimated by the use of PI. In the existing view [2], the parameter PI adequately describes cell proliferation only if only one of the two daughter cells retain the ability to divide, a characteristic feature of differentiating tissues in the body. Under conditions of cell culture, however, the author cited considers that the parameter N is more appropriate. Considering the linear character of the change in the two parameters depending on the cell fixation time, the mean doubling time of the cell population can also be calculated for any two cell fixation points, by the equation:

$$T = \frac{t' - t}{P_{t'} - P_t},$$

where t' and t denote the cell fixation times and P' and P are parameters of proliferative activity at the later and earlier fixation points, respectively. Due to stochastic processes, assessment of the kinetics of cell proliferation for two fixation points may be reliable if each point is well "loaded" and the principle of identical conditions of cell culture is observed.

To verify the results, in the experiments of series II when lymphocyte cultures from 20 donors were fixed after 72 and 90 h of culture (Table 2) the calculated values of PI were 1.51 and 2.26, and of N 1.34 and 1.79, respectively. Estimation of the average doubling time of the cell population by the equation given above gives values of 24 and 40 h for PI and N respectively. These same values also were obtained from regression equations in the form: $PI = -1.4900 + 0.0417 \cdot t$ and $N = -0.4600 + 0.0250 \cdot t$. These estimates of the average doubling time of the cell population by means of the corresponding parameters were close to values found in the experiments of series I, but the values of the time of emergence of the first cells from the G_0 stage differed significantly (Table 3). Consequently, when lymphocyte cultures are grown in medium 199 the cells emerge from the G_0 stage more slowly than cells growing in Eagle's medium, whereas the mean doubling time of the cell population is independent of the conditions of culture, and is 24-25 h with respect to PI and 38-40 h according to N for healthy blood donors aged 10-19 years. The stability of the mean doubling time of the cell population under different conditions of cell culture reflects stability of the mean duration of the cell cycle of lymphocytes.

Incidentally, the figure of 12-14 h obtained by some workers [9, 11] for the duration of the cell cycle of lymphocytes is only half of the average doubling time of the cell population calculated from the parameter PI. This result requires further verification. It must be emphasized that the estimate obtained for the average doubling time of the lymphocyte population for a group of individuals does not rule out the possibility of interindividual variations. In addition, the duration of the cell cycle may vary, both under normal conditions (for example, during aging [15]) and in some pathological states [6, 7].

The suggested method of evaluating proliferative activity by the use of two cell fixation points enables variations of the kinetics of cell proliferation under normal and pathological conditions to be studied more accurately than by the uninformative analysis of the simple ratio between mitoses at one fixation point.

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