DETERMINATION OF THE PROLIFERATIVE POOL AND DURATION OF THE MITOTIC CYCLE IN COLONIES OF FIBROBLAST-LIKE CELLS IN MONOLAYER BONE MARROW CULTURES

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The proliferative pool of fibroblasts in 7-day and 12-day explants of guinea pig bone marrow under monolayer cultivation conditions was below 100%, amounting to about 88% for 7-10 day, cultures and 78% for 12-15 day cultures. The duration of the mitotic cycle of the fibroblasts was about 40 h, and the duration of the S-period was one-quarter of this time, i.e., 8-10 h.

It was shown previously [2-4] that during monolayer cultivation of guinea pig spleen and bone marrow cells, discrete colonies or clones, consisting of fibroblasts, are formed in the cultures. Toward the 10th day of cultivation these colonies become large enough to be seen with the naked eye, and they continue to grow intensively until they fuse into a continuous monolayer.

The objective of the present investigation was to determine the duration of the mitotic cycle of the cells in the colonies with the aid of thymidine-H^3 saturation curves and to determine the proliferative pool in the colonies.

EXPERIMENTAL METHOD

A suspension of bone marrow cells from the femora of guinea pigs (weighing 100-200 g) was prepared as described previously [3]. The resulting suspension was grown in penicillin flasks with cover slips in a medium of the following composition: medium No. 199 80%, bovine serum 20%, penicillin and streptomycin 100 units/ml of each. Each flask was charged with $4 \cdot 10^6$-$6 \cdot 10^6$ nucleated marrow cells in 2 ml medium.

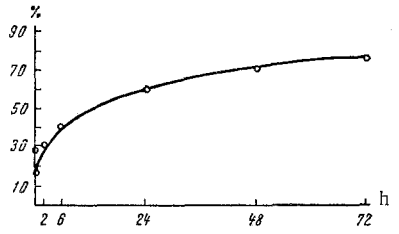

Fig. 1. Curve of saturation with thymidine-H^3 of fibroblasts forming foci in 12-day cultures of guinea pig bone marrow. Abscissa, time of incubation of cultures in medium containing thymidine-H^3 (in h); ordinate, number of labeled fibroblasts (in %).

The first change of medium took place 24 h after transplantation, and it was subsequently changed every 3-4 days as its pH changed.

On the 7th and 12th days of cultivation, at the same time as the medium was changed, thymidine-H^3 with a specific activity of 1.9 Ci/mmole was added to the cultures in a concentration of 1 μCi/ml. Later, thymidine-H^3 was added to the culture medium once daily.

At various times after addition of the isotope, the cultures were washed and fixed with 96° ethanol. After treatment with 3% perchloric acid, the cultures were coated with type M liquid

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TABLE 1. Relationship between Number of Labeled Fibroblasts in 7-Day Cultures and Duration of Incubation with Thymidine-H^3

	Duration of incubation with thymidine-H^3 (in h)												
	0,25	0,5	2	4	8	16,5	20	24	28	32	41	48	68
No. of labeled cells (in %)	18	27	30	39	40	42	50	66	75	90	86	89	86
Standard deviation (σ)	3	3	6,3	2	2	32	5	7,2	14,7	1	5	1	3

TABLE 2. Relationship between Number of Labeled Fibroblasts in 12-Day Cultures and Duration of Incubation with Thymidine-H^3

	Duration of incubation with thymidine-H^3 (in h)						
	0,25	0,5	2	6	24	48	72
No. of labeled cells (in %)	18,2	29,2	31,3	40	59	73	78
Standard deviation (σ)	4,2	1,1	2	7,1	8,6	5,6	1,4

TABLE 3. Determination of Duration of Mitotic Cycle of Fibroblast-Like Cells in 7-10 Day Cultures (N_p=88%; N_S=18%).

Δt (in h)	ΔN (in %)	a	T (in h)
3,5	8	0,019	36,3
4	6	0,0125	55,2
4	15	0,031	22,6
15,5	48	0,026	26,2
11,5	33	0,025	27,6
7,5	14	0,017	44,5
8	25	0,026	26,5
12	40	0,028	24,6
8	34	0,0355	19,4
4	19	0,038	18,2

T_{mean}=30.1 h, σ=3.6.

Note. For meanings of letters in Tables 3 and 4, see text.

TABLE 4. Determination of Duration of Mitotic Cycle of Fibroblast-Like Cells in 12-Day Cultures (N_p=78%; N_S=18%).

Δt (in h)	ΔN (in %)	a	T (in h)
1,5	2,1	0,012	53,0
4	8,7	0,020	34,5
5,5	10,8	0,018	38,3
25,5	29,8	0,017	40,6
2,2	27,7	0,013	53,0

T_{mean}=43.4 h; σ=4.4.

emulsion and exposed for 5 days. After development, the autoradiographs were stained with Carazzi's hematoxylin, and the percentage of labeled fibroblasts and, in some cases, the percentage of labeled histiocytes also, were estimated in the foci. Cells were regarded as labeled if they contained no fewer than five silver grains over their nuclei, above the background level, but as a rule the intensity of labeling was far higher than this. In each specimen 200-500 cells were counted, and altogether about 100 explants were used.

The duration of the mitotic cycle of the fibroblasts was determined by means of approximate equations developed for an exponentially growing cell population [1]:

$$T = \frac{\ln 2}{a},$$

where $a = \Delta N/(N_S + N_p)\Delta t$; T is the duration of the cycle; N the increase in number of labeled cells (in %) during time Δt; N_S the percentage of cells in the S-period (i.e., incorporating thymidine-H^3 during isotope labeling); and N_p the size of the proliferative pool (in %).

EXPERIMENTAL RESULTS

In the 7-day cultures the saturation of the fibroblasts in the foci with thymidine-H^3 reached a maximum between 28 and 32 h. With a further increase in the duration of incubation with thymidine-H^3 the number of labeled cells did not increase but remained at a mean level of 88% (Table 1). By the 12th day of cultivation the proliferative pool of this population in the foci consisting of fibroblasts was a little smaller namely about 78% (Fig. 1; Table 2), and saturation was reached after about 48 h.

The length of the mitotic cycle of the fibroblast-like cells, determined on the basis of the results in Tables 3 and 4, was about 30 h for the 7-day cultures and about 43 h for the 12-day cultures.

The saturation curve for the 7-day cultures reached a plateau after 32 h. This period of time must correspond to $T-T_S$ (where T is the duration of the mitotic cycle and T_S the length of the S-period). The proportion of cells in the S-period (N_S) is equal to the isotope labeling index. If incubation with thymidine-H^3 for 15 min is equated with isotope labeling, N_S for the fibroblasts in the colonies is 18% (Tables 1 and 2). According to Quastler's equation [5], $N_p/T = N_S/T_S$, whence $T_S = (N_S/N_p)\,T = 18\%/88\% \cdot T = 20\%\ T$. Sine $T-T_S$=32 h, we obtain T=40 h.

Hence the duration of the mitotic cycle (T), determined on the basis of the time taken for the saturation curve (Table 1) to reach a plateau, is 40 h.

In some cases the percentage of labeled histiocytes also was determined. This category included cells similar to macrophages, histiocytes, and reticulum cells in their morphology. With increasing age of the cultures the proliferative activity of the histiocytes decreased appreciably. For instance, in the first 24 h, 23.2% of the histiocytes were labeled, but only 10.9% in the second day, 8% in the third, and 1.5% in the sixth day.

The rapid increase in size of the colonies in the course of cultivation suggested that the cells in the colonies were in a state of active proliferation [2]. A study of thymidine-H^3 incorporation confirmed this suggestion. In 7-day cultures with an isotope labeling index of 18%, saturation occurred after 32 h, when the labeling index had reached 90%. In 12-day cultures with the same isotope labeling index, saturation occurred after about 48 h (labeling index at saturation 78%).

The mean duration of the mitotic cycle of the fibroblasts in the colonies, determined from the saturation curves, was about 30 h for the 7-day cultures and about 40 h for the 12-day cultures, in agreement with the results obtained by measuring the time required for the number of fibroblasts in the foci to double itself [2].

The fact that the proliferative pool in the 7- and 12-day cultures was less than 100% and that it was somewhat lower in the 12-day than in the 7-day cultures, may indicate that some cells in the colonies abandon the state of proliferation as the colonies grow. This question requires special examination.

The number of fibroblast-like cells incorporating thymidine-H^3 during isotope labeling (15 min) and the size of the proliferative pool of the population can be used to estimate the duration of the S-period of these cells. It was found that the approximate duration of the S-period is one-quarter of the total duration of the cycle, i.e., 8-10 h.

The proliferative activity of the histiocytes falls sharply with increasing age of the cultures, and this is accompanied by a parallel decrease in the relative proportion of histiocytes among the cultivated cells.

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