CLONE FORMATION IN MONOLAYER CULTURES OF BONE MARROW AND SPLEEN

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Cultures of guinea pig bone marrow and spleen were used to study the relationship between the number of foci of fibroblast-like cells formed and the number of cells cultivated, and also to examine the structure of the focus-forming unit. X and Y chromosomes were used as markers.

* * *

A gradual interchange of cell populations takes place in monolayer cultures of bone marrow and spleen cells [2-4, 6, 7]. In the first few days the cultures contain hematopoietic cells, leukocytes (which gradually degenerate), and histiocytes (giving off numerous processes). After the 3rd-10th day histiocytes are most numerous in the cultures, but solitary large fibroblast-like cells with an oval nucleus and pale cytoplasm, filled with tonofibrils, begin to appear. The number of histocytes in guinea pigs starts to decrease from this time, and by the 15th-20th day they have practically disappeared. To replace them, starting from the 10th day, discrete colonies consisting of fibroblast-like cells, with the structure described above, develop in the cultures (Fig. 1). These foci grow in size continually and may merge to form a continuous monolayer.

The object of this investigation was to determine the nature of these foci or, more specifically, to discover whether they are cell clones or whether they are formed by associations of cells originally lying separately. Two methods were used to study this problem.

EXPERIMENTAL METHOD

The relationship between the number of foci formed and the number of cells attached to the slides was studied. In each experiment bone marrow or spleen cells from one animal were used. Cells of organs taken from adult guinea pigs were suspended in medium No. 199 with 20% heated bovine serum, and after careful filtration through kapron they were placed in 100 ml flat-bottomed culture flasks. Each flask, containing 12 ml culture medium, was seeded with a different number of cells ($1.2 \cdot 10^6$ to $48 \cdot 10^5$). The cultures were incubated at 37°. The medium was changed after 24 h and the number of decanted (unattached) cells in it counted. On the 12th-15th day the cultures were washed to remove serum, fixed in 96° alcohol, and stained with hematoxylin, after which the number of cells consisting of fibroblast-like cells was counted. By this time the histiocytes and hematopoietic cells had practically disappeared from the cultures.

Spleen cells from two guinea pigs reared under sterile conditions also were used for cultivation. These animals were obtained at the age of 3.5 months from Senior Scientific Assistant O. V. Chakhava at the N. F. Gamaleya Institute of Epidemiology and Microbiology.

In the second method equal numbers of spleen cells from a male and female were filtered through kapron, pooled in vitro, and cultivated in Leighton tubes in the bottom of which cover slips were placed. Each tube contained $3 \cdot 10^6$-$5 \cdot 10^6$ cells in 1.5 ml medium. The presence of X and Y chromosomes in the metaphase plates was determined in discrete foci grown on cover slips, after treatment with colchicine and subsequent conversion by a modified Ford's method [1] into total preparations.

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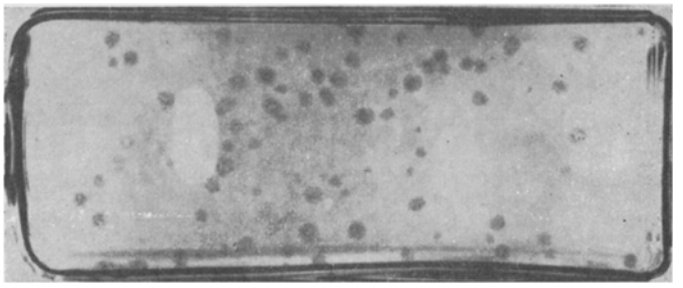

Fig. 1. Culture of guinea pig bone marrow cells (15 days). Hematoxylin.

TABLE 1. Relationship between Number of Foci of Fibroblast-Like Cells in Culture and Number of Attached Cells

Tissue cultivated	No. of attached cells	No. of foci on 12th day	FFU	Mean FFU	Deviation from mean FFU
Spleen	400 000	7	$0,5 \cdot 10^5$	$0,6 \cdot 10^5$	$-0,1 \cdot 10^5$
	500 000	10	$0,5 \cdot 10^5$		$-0,1 \cdot 10^5$
	8 800 000	87	$1,0 \cdot 10^5$		$+0,4 \cdot 10^5$
	17 100 000	320	$0,5 \cdot 10^5$		$-0,1 \cdot 10^5$
	18 000 000	350	$0,5 \cdot 10^5$		$-0,1 \cdot 10^5$
	37 300 000	550	$0,6 \cdot 10^5$		0
	38 800 000	565	$0,6 \cdot 10^5$		0
	4 100 000	7	$5,8 \cdot 10^5$	$2,4 \cdot 10^5$	$+3,4 \cdot 10^5$
	3 950 000	9	$4,2 \cdot 10^5$		$+1,9 \cdot 10^5$
	5 850 000	36	$1,6 \cdot 10^5$		$-0,8 \cdot 10^5$
	4 700 000	41	$1,1 \cdot 10^5$		$-1,3 \cdot 10^5$
	26 950 000	148	$1,8 \cdot 10^5$		$-0,6 \cdot 10^5$
	27 450 000	157	$1,7 \cdot 10^5$		$-0,7 \cdot 10^5$
	21 150 000	122	$1,7 \cdot 10^5$		$-0,7 \cdot 10^5$
	22 550 000	151	$1,5 \cdot 10^5$		$-0,9 \cdot 10^5$
Bone marrow	1 000 000	4	$2,2 \cdot 10^5$	$1,9 \cdot 10^5$	$+0,3 \cdot 10^5$
	900 000	2	$4,0 \cdot 10^5$		$+2,1 \cdot 10^5$
	19 000 000	182	$1,0 \cdot 10^5$		$-0,9 \cdot 10^5$
	19 000 000	162	$1,0 \cdot 10^5$		$-0,9 \cdot 10^5$
	32 300 000	190	$1,5 \cdot 10^5$		$-0,4 \cdot 10^5$
	35 200 000	120	$2,6 \cdot 10^5$		$+0,7 \cdot 10^5$
	950 000	6	$1,5 \cdot 10^5$		$-0,3 \cdot 10^5$
	900 000	4	$2,2 \cdot 10^5$		$+0,4 \cdot 10^5$
	3 350 000	16	$2,0 \cdot 10^5$		$+0,2 \cdot 10^5$
	3 100 000	12	$2,5 \cdot 10^5$		$+0,7 \cdot 10^5$
	7 150 000	55	$1,3 \cdot 10^5$		$-0,5 \cdot 10^5$
	6 660 000	40	$1,6 \cdot 10^5$		$-0,2 \cdot 10^5$
	6 800 000	48	$1,4 \cdot 10^5$	$1,8 \cdot 10^5$	$-0,4 \cdot 10^5$
	13 900 000	80	$1,7 \cdot 10^5$		$-0,1 \cdot 10^5$
	13 200 000	65	$2,0 \cdot 10^5$		$+0,3 \cdot 10^5$

EXPERIMENTAL RESULTS

The results of counting the number of foci and its relationship to the number of attached cells in four experiments are given in Table 1. This table shows the number of foci in the separate flasks of each experiment and the corresponding size of the focus-forming unit (FFU), i.e., the number of attached cells contained in the focus. The mean size of the FFU and the magnitude of the deviation from it for the individual flasks are given for each experiment. It will be noted that the scatter of FFU in each experiment, except for single points, was less than the difference between the mean values of FFU in different experiments. Because of this, two experiments were carried out. In the first of them a suspension of bone marrow cells was distributed among 19 flasks as follows: into 9 flasks in concentrations of $2 \cdot 10^6$–$3 \cdot 10^6$, into 5 flasks in concentrations of $7 \cdot 10^6$ to $8 \cdot 10^6$, and into 5 flasks in concentrations of $22 \cdot 10^6$ to $25 \cdot 10^6$. The same procedure was repeated in the second experiment with a suspension of spleen cells. The results obtained by cultivation of spleen cells from sterile guinea pigs are given in Fig. 3.

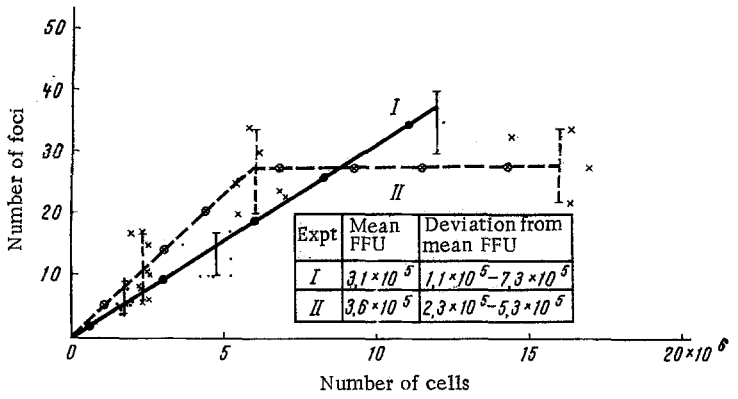

Fig. 2. Number of foci of fibroblast-like cells during cultivation of the same cell suspension in different concentrations: I) bone marrow; II) spleen.

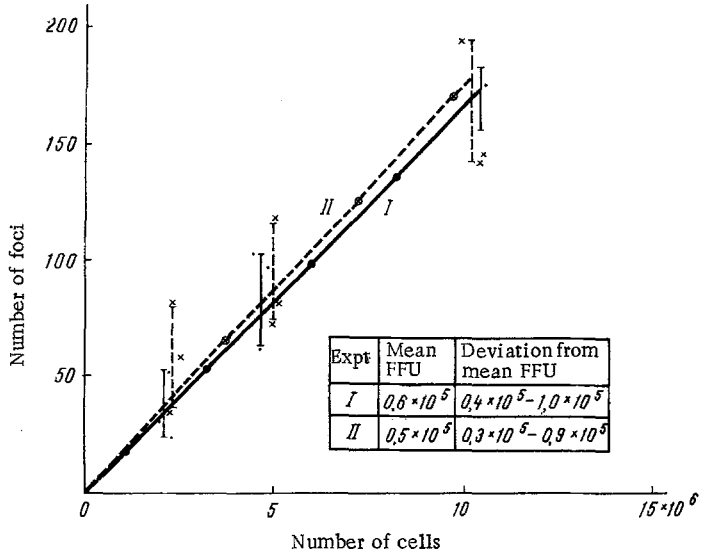

Fig. 3. Number of foci of fibroblast-like cells grown from spleen cells of guinea pigs reared under sterile conditions: I) spleen of first guinea pig; II) spleen of second guinea pig.

Considerable difficulty was experienced in the counting of X and Y chromosomes in metaphase plates of the fibroblast-like cells, during preparation of total preparations suitable for chromosome analysis. Although in each experiment many cells were in a state of mitosis (several dozens, in fact), only 10 foci in which between 3 and 5 plates could be identified were analyzed. Each plate was photographed. The distribution of cells from male and female is given in Table 2, It follows that all ten investigated foci contained dividing cells of the male or the female only.

It may be that foci consisting of fibroblast-like cells, which appeared on the 10th-15th day of cultivation of guinea pig bone marrow and spleen cells, are formed on account of a few focus-forming units distributed uniformly among the cells of a given population. It is thereby assumed that the foci are formed independently. If this hypothesis is correct, the distribution of the number of foci must be close to Poisson in type if the number of cells taken from the given population is identical.

This hypothesis was tested in an experiment in which bone marrow cells taken from one animal in a concentration of $2 \cdot 10^6$ cells per flask were cultivated in 9 flasks. Besides determining the FFU for each flask, in this experiment the number of foci was reduced to the mean number of attached cells for the given

TABLE 2. Chromosome Analysis of Composition of Foci of Fibroblast-Like Cells

Serial	No. of metaphase plates identified in focus	Male / Female
1	4	0/4
2	4	0/4
3	5	5/0
4	3	3/0
5	3	0/3
6	4	4/0
7	5	5/0
8	3	0/3
9	5	0/5
10	4	0/4

concentration. The results of this experiment were subjected to statistical analysis. The Poisson hypothesis was tested by the χ^2 criterion; satisfactory agreement was obtained for this particular experiment; it could not be used in the other experiments because of the small size of the samples.

When spleen cells from sterile guinea pigs were cultivated the differences between the mean values of FFU in these two experiments were smaller than the corresponding difference for nonsterile animals.

So far as the structure of the FFU is concerned, this could consist either of one cell, in which case each focus was a cell clone, or foci could be formed as the result of cell aggregation. In this case the FFU consisted of several cells. Chromosome analysis of 10 foci confirmed the validity of the first hypothesis. In fact, all 10 investigated foci contained dividing cells either from the male only or from the female only. This is in agreement with the linear growth of the number of foci with an increase in the number of transplanted cells. As the graph (Fig. 2) shows, this relationship holds good if there were not more than 10^7 cells per 100 ml flask. If more cells were transplanted, the linear increase in the number of cells was disturbed, probably because of an excessive density of cells on the surface of the flask. The concentration of focus-forming cells discovered under the conditions of cultivation used in these experiments was 10^5 for bone marrow and spleen cells. The foci of fibroblast-like cells developing in cultures of guinea pig bone marrow and spleen cells were thus cell clones formed as a result of multiplication of focus-forming cells. It must be supposed that not all the focus-forming cells (i.e., precursors of fibroblasts) reveal themselves by forming colonies in vitro. The actual concentration of these cells in hematopoietic tissue is evidently, therefore, higher. In mouse bone marrow one stem cell, detected by the method of splenic colonies in vivo, is present to 10^4 of the other cells [5]. It is too early as yet to suggest how, with respect both to their morphology and origin, the cells of hematopoietic tissue transferred to the cultures when washed out of the bone marrow and spleen, play the role of focus-forming cells. However, they are certainly fewer in number than the precursor cells of the macrophages. These two categories of precursor cells are, therefore, evidently not identical.

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