MICROFLUOROMETRIC STUDY OF ERYTHROBLASTIC ISLETS AND MACROPHAGES OF BONE MARROW

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Cellular-humoral interactions in the regulation of erythropoiesis are realized in erythroblastic islets (EI) of the bone marrow, which constitute an association of a macrophage (Mph) with its surrounding erythroid cells (EC) in various stages of differentiation. EI are considered to be morphological and functional units of erythropoiesis, in which Mph performs various functions in relation to EC: creation of affinity for precursor cells of erythropoiesis (CFV_e), ensuring a specific microenvironment, and trophic and phagocytic [5, 13] functions. Methods used to study the functional state of cells, the rate of proliferation of EC, and activity of the lysosomal complex of Mph, have not been adequately developed for living EI [13]. The use of intravital staining of EI with a metachromatic fluorochrome – acridine orange (AO) – seems promising, for it has been shown that the monomeric form of AO in living cells binds mainly with native DNA and gives green fluorescence, whereas in lysosomes AO accumulates selectively in the form of red cytoplasmic granules [6, 15]. These methods are used to assess the functional state of Mph and the character of the course of internal diseases, including hematologic diseases [8, 9, 12]. Microfluorometry, which can be used to determine the lysosomal and biosynthetic activity of living cells, could provide new information on mutual relations between the lysosomal apparatus of Mph and biosynthetic processes in EC of the erythroblastic islets. However, we could find no reference to studies of these characteristics on the basis of microfluorometry in EI of different stages of maturity, under normal conditions and after stimulation by erythropoietin. The investigation described below was undertaken in order to solve this problem.

EXPERIMENTAL METHOD

Experiments were carried out on 27 intact Wistar rats weighing 180-200 g, of both sexes. A suspension of bone marrow cells containing EI and Mph was obtained as described previously [5]. Intravital preparations fluorochromed with AO were obtained by the method in [11] and studied on the "Lyumam-I3" microscope. Microfluorimetry of EI was carried out with the FMÉL-1A attachment with FÉU-79 photoelectric multiplier, with excitation from above with blue light from an OI-35 source put in place of the light source with DRSh 250-3 lamp, and with a KGM-70 incandescent lamp with voltage stabilized within 0.1% [7]. Spontaneous movement of the luminescent filament in gas-discharge tubes, even with strict stabilization, interferes with the obtaining of reproducible data [1, 7]. When recording fluorescence it is recommended that the intensity of the exciting light be reduced [10]. As exciting filter we used the SS-15-2 filter used previously in similar investigations [2]. Recording was carried out for

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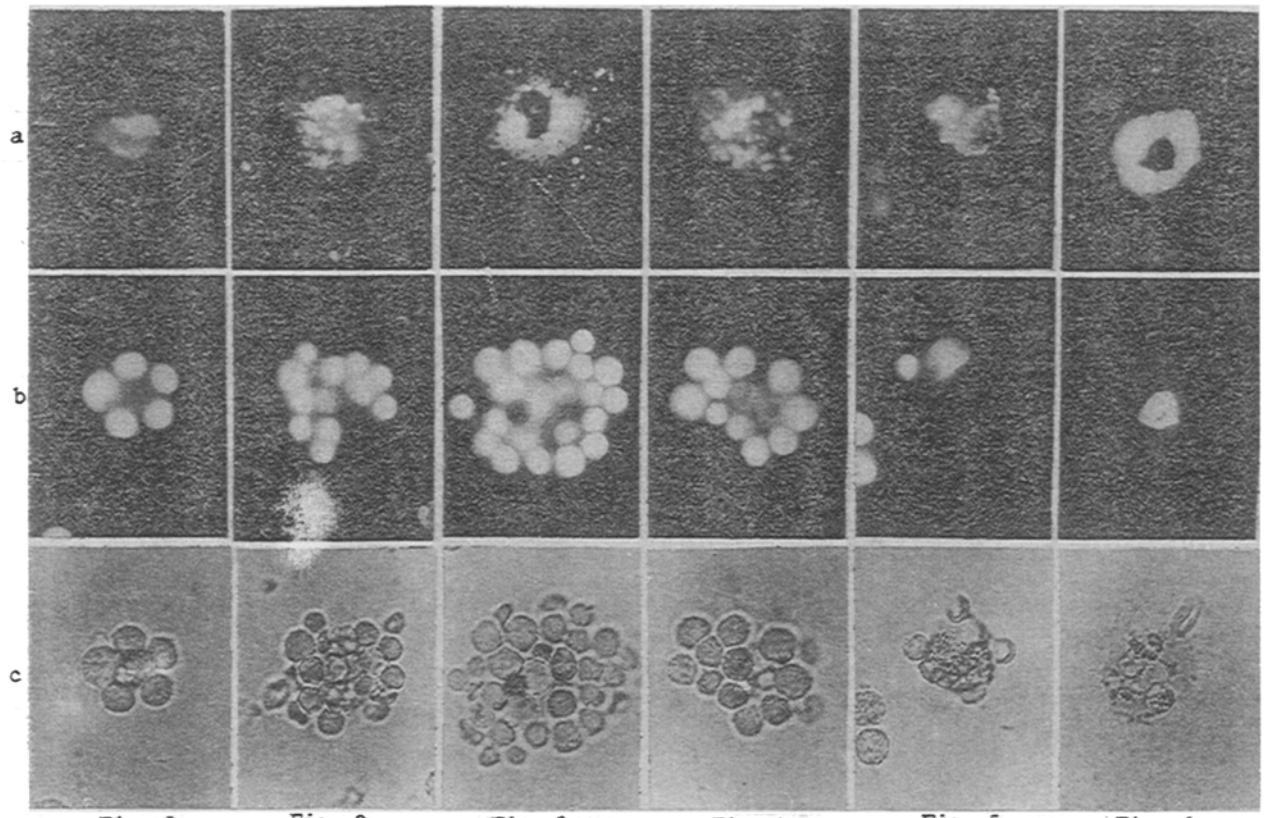

Fig. 1 Fig. 2 Fig. 3 Fig. 4 Fig. 5 Fig. 6

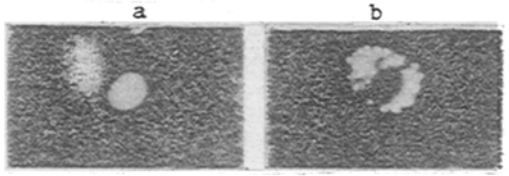

Fig. 7

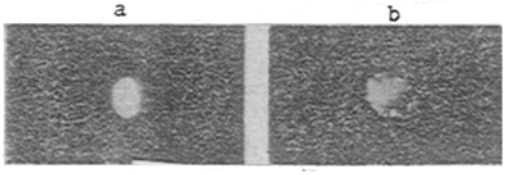

Fig. 8

Fig. 1. EI of maturity class I. No reticulocytes present in corona of macrophage. In Figs. 1-8) Erythroblastic islets (EI) and macrophages of rat bone marrow, fluorochromed with acridine orange, adherent to glass. Objective 70×, ocular 7×. a) Isolation of green fluorescence, b) red, c) under light microscope.

Fig. 2. EI of maturity class II.

Fig. 3. EI of maturity class III.

Fig. 4. Reconstructed EI.

Fig. 5. Involuting EI.

Fig. 6. Involuting EI. Only reticulocytes present in corona of macrophage.

Fig. 7. Macrophage with intensely fluorescent lysosomal granules in cytoplasm, probably with affinity for erythroid tissue.

Fig. 8. Macrophage with weakly fluorescent lysosomal granules in cytoplasm.

TABLE 1. Proliferative Processes in EI and Activity of Lysosomal Apparatus of Mph in EI (data of microfluorometric investigation; M ± m)

Maturity class [5]	Number of reticulocytes	Intensity of fluorescence				Involution index of EI
		630-650 nm (lysosomes)		530-550 nm (DNA)		
		total	specific	total	specific	
Involuting, having only reticulocytes	n=14 5,14±0,79	n=14 1,27±0,19	n=14 0,26±0,03	n=14 0,61±0,09	—	n=14 2,33±0,29
Reconstructed – with reticulocytes and proerythroblasts, and with vasophilic normoblasts	n=72 4,69±0,30	n=72 1,76±0,08	n=72 0,52±0,05	n=72 1,33±0,07	n=72 0,60±0,03	n=72 1,42±0,05
I – up to 8 nucleated cells	—	n=20 1,72±0,19	—	n=20 2,40±0,27	n=20 0,64±0,06	n=20 0,88±0,14
II – 9-16 nucleated cells	n=108 4,34±0,30	n=108 2,32±0,09	n=99* 0,64±0,04	n=108 4,60±0,17	n=108 0,41±0,01	n=108 0,53±0,02
III – over 16 nucleated cells	n=11 4,36±0,94	n=11 3,49±0,29	n=10** 1,00±0,21	n=11 7,82±0,73	n=11 0,39±0,04	n=11 0,46±0,03
Involuting – with orthochromic normoblasts and reticulocytes	n=275 3,92±0,14	n=275 1,55±0,04	n=275 0,53±0,02	n=275 2,19±0,06	n=275 0,45±0,01	n=275 0,76±0,02

Legend. *) Nine islets were disregarded because no reticulocytes were in the corona of their Mph; **) reticulocytes not present in corona of Mph in one islet.

TABLE 2. Effect of Erythropoietin on Quantity of DNA-Containing Material and Lysosomal Activity in Erythroblastic Islets of Bone Marrow (data of microfluorometric investigation)

Group of observations		Incubation for 30 min (5 rats)		p	Incubation for 60 min (5 rats)		p
fluorescence spectrum, nm	parameter	control	experiment		control	experiment	
630—650 (Lysosomes)	n	110	110		110	110	
	M	1,48	2,04		1,49	1,45	
	m	0,08	0,10		0,07	0,08	
	δ	0,81	1,05	<0,01	0,71	0,81	
530—550 (DNA)	n	110	110		110	110	
	M	1,95	2,42		1,92	1,34	
Involution of index	m	0,16	0,20		0,11	0,08	
	δ	1,67	2,10		1,19	0,88	<0,01
	n	110	110		110	110	
	M	0,99	1,20		1,00	1,18	
	m	0,05	0,06		0,04	0,05	
	δ	0,5	0,66	<0,01	0,45	0,57	<0,05

Legend. n) Number of erythroblastic islets: p for comparison of control and experimental tests.

2-3 sec on the SHCH 4300 instrument in the 200 V range, with supply voltage of 2200 V to the FÉU-79 instrument, probe diameter 1.5 mm, and objective 40 × 0.75 (water immersion). Fluorescence was selected in spectral bands 530-550 nm and 630-650 nm, using FMÉL No. 10 and No. 15 interference filters and light-dividing plates with ZS-11 and KS-11 filters. The intensity of background fluorescence during recording in the 200-V band of the instrument was zero, but in the 20 V band it was 0.02-0.04 conventional unit (C.U.). Selectivity of the filters was controlled visually. Nucleated EC and ER were counted and the number of reticulocytes found is the difference between the total number of EC in ER and the number of nucleated EC. Total intensity of fluorescence (TIF) in the green part of the spectrum, indicating the DNA content in EI, was divided by the number of nucleated EC, and TIF in the red part of the spectrum was estimated as an indicator of activity of the lysosomal apparatus of Mph in EI, and divided by the number of reticulocytes in EI.

Microfluorometry of bone marrow Mph, smaller in area than those in EI, was carried with a 65 × 1.1 objective (water immersion) and the supply voltage of the FÉU-79 was reduced to 2000 V. The remaining parameters of the conditions described above were left unchanged.

EI were arranged in classes of maturity [5].

When the effect of erythropoietin (Bp) on the intensity of fluorescence in the cell components of EI which was added in a dose of 1 U in 0.02 μl to the incubation medium. The Ep was obtained from human urine in Terry Fox's Laboratory, Vancouver, Canada, and titrated at the Peterson Institute, Manchester, England, and presented to Professor Yu. M. Zakharov.

EXPERIMENTAL RESULTS

Altogether 500 EI from bone marrow of 13 rats were tested. Within the range 530-550 nm the average TIF was 2.65 ± 0.08 C.U. The specific intensity (SIF 530), obtained as the quotient by dividing TIF 530 by the number of nucleated EC in EI, in 486 EI was 0.47 ± 0.01 C.U. The remaining 14 E.I. had no EC with nuclei in their composition and continued to have only reticulocytes. In the 630-650 nm range TIF averaged 1.78 ± 0.04, and SIF 630, obtained by dividing TIF 630 by the number of reticulocytes in an islet, in 470 EI was 0.55 ± 0.02 C.U. No reticulocytes were found in 30 EI.

As an additional criterion for assessing the relationship between lysis and proliferation in EI, the ratio of TIF 630 to TIF 530, which has been called the involution index (II) of EI, was used.

Among the involuting EI the 14 EI (2.8%) with only reticulocytes in their composition, the number of which varied from 3 to 10, formed a special group (Fig. 6). In fact, the parameters of TIF obtained were characteristics of Mph of an EI which performed the function of maintaining the erythroid clone of EI and which, it is considered, can make contact with a new CFU_e. EI of this type were characterized by the lowest values of TIF and SIF and the highest values of II. Reconstructed EI (14.4%), with both reticulocytes and juvenile EC in their composition, revealed a high level of SIF 530, comparable with the same parameter of EI of maturity class I, consisting of proliferating EC only. The reconstructed EI were also characterized by quite high values of II (Fig. 4).

In several KI of maturity classes I (4%), II (21.6%), and III (2.2%) the increase in number of nucleated EC was accompanied by a regular increase of TIF in both parts of the spectrum and by a decrease in II, reflecting an increase in the intensity of erythropoiesis in EI (Figs. 1-3). However, whereas SIF 630 also increased, indicating intensification of the phagocytic function of the Mph of EI, SIF 530, on the contrary, fell successively, evidence of weakening of the proliferative powers of the maturing EC. The most numerous group among EI was the involuting islets (55%), which contained fewer than eight nucleated EC in the late stages of development and reticulocytes, with TIF 630 and SIF 630 values close to those of involuting EI, containing only reticulocytes, and also to those of reconstructed (Fig. 5). In relation to II the islets of this group occupied an intermediate position between EI with activately proliferating EC and EI in which utilization of phagocytosed material by Mph was the predominant process.

Considering the correlation between TIF and SIF, on the one hand, and the state of erythropoiesis in EI of different maturity classes on the other hand, the effect of Ep, which is a hormone of erythropoiesis, on these parameters was studied in the next series of experiments. Suspensions of bone marrow from 10 rats were used to set up control and experimental tests. The data in Table 2 show that exposure to Ep for 30 min of incubation led to activation of the lysosomal apparatus of Mph of EI with an increase in TIF 630 of EI by 38% and an increase in II of Er by 21%. As a result of exposure for 60 min to Ep, TIF 530 of EI decreased by 30%, most probably due to dissociation of nucleated erythroid cells of EI. The cause of this could be increased secretion of lysosomal enzymes by Mph of EI, as indicated by a decrease of 1.4 times ($p < 0.001$) in TIF 630 during incubation for 60 min compared with TIF 630 after incubation of EI with erythropoietin for 30 min. This hypothesis requires additional experimental confirmation. However, the high rate of digestion of expelled normoblast nuclei by Mph of EI is evidence in support

of this suggestion [16]. Finally, proteinases of macrophagal origin are known to carry out enzymic destruction of the intercellular matrix [12]. Brief exposure to Ep in our experiments was not accompanied by activation of proliferation of EC, which peaked 24 h after exposure to EP. Sensitivity of Mph of EI to the action of Ep, discovered by microfluorometry, is a fact of fundamental importance, for it had been considered that only CFU_e and EC possess this property. Perhaps the action of EP in EI is mediated through the central Mph.

In the next series of experiments an attempt was made to characterize the bone marrow Mph subpopulation with affinity for erythropoiesis in terms of TIF 630 and TIF 530. Assuming that the high values of II found in involuting EI with reticulocytes and in EI during incubation with Ep may be characteristic of Mph that are capable of forming EI, we studied 200 bone marrow Mph from four rats, and on the basis of the calculated II we divided them into two groups. In the 1st group (101 Mph) II varied from 2.0 to 6.5, with a mean value of 3.12 ± 0.11 (Fig. 7). In the 2nd group (99 Mph) II varied from 0.3 to 1.8, with a mean value of 1.09 ± 0.04 (Fig. 8). The two groups of Mph differed significantly with respect to TIF also. For instance, TIF 530 for Mph of the 1st group was 0.31 ± 0.01, and of the 2nd group 0.48 ± 0.01 C.U. ($p < 0.001$). Conversely, TIF 630 for Mph of the 1st group was 0.92 ± 0.04, and of the 2nd group 0.53 ± 0.02 C.U. ($p < 0.001$). The bone marrow Mph population is evidently heterogeneous. On the basis of the pooled data of the three series of experiments, Mph of the 1st group can be classed as potential Mph of EI. High values of II and TIF 630 for these Mph probably reflect their permanent role in phagocytosis of the expelled normoblast nuclei, and also their ability to modify their functional activity under the influence of Ep.

This intravitral microfluorometric study of bone marrow EI, undertaken for the first time, thus revealed the same patterns as were found by cytochemical and morphological methods [13, 16]. Accumulation of nucleated cells in the corona of EI is combined with increased activity of macrophagal lysosomes of erythroblastic islets. Meanwhile the method we used, which enables the time course of relations between processes of cell proliferation and activity of Mph in EI to be described, also revealed a fundamentally important characteristic of EI, namely high values of the ratio of lysosomal activity to DNA. According to this characteristic the macrophagal subpopulation of EI differs from that of other bone marrow Mph. These findings, in our view, prove that it is correct to distinguish an Mph subpopulation with affinity for the erythroid series in hematopoietic tissue.

REFERENCES

1. L. S. Agroskin and G. V. Papayan, Cytophotometry [in Russian], Leningrad (1977).
2. Yu. P. Antipuchuk and S. B. Koval', Tsitologiya, **24**, No. 7, 844 (1982).
3. Yu. M. Zakharov, I. Yu. Mel'nikov, and A. G. Rassokhin, Gematol. Transfuziol., No. 4, 52 (1984).
4. Yu. M. Zakharov and I. Yu. Nel'nikov, Gematol. Transfuziol., No. 10, 51 (1984).
5. Yu. M. Zakharov, I. Yu. Mel'nikov, and A. G. Rassokhin, Hematopoietic Precursor Cells [in Russian], Chelyabinsk (1986), pp. 20-21.
6. A. V. Zelenin, Interaction between Amino Derivatives of Acridine and the Cell [in Russian], Moscow (1971).
7. G. V. Papayan, V. A. Ioffe, G. N. Vinogradova, and I. Ya. Barskii, Tsitologiya, **16**, No 3, 395 (1974).
8. I. S. Podosinnikov, I. V. Babachenko, and A. N. Zakharov, Lab. Delo, No. 8, 451 (1985).
9. T. L. Rudakova and L. I. Nikolaeva, Gematol. Transfuziol., No. 5, 28 (1984).
10. A. D. Sorkin and L. V. Teslenko, Methods of Cell Culture [in Russian], Leningrad (1988), pp. 126-136.
11. I. Ya. Uchitel', Macrophages in Immunity [in Russian], Moscow (1983).
12. I. S. Freidlin, The Mononuclear Phagocyte System [in Russian], Moscow (1984).
13. M. Bessis, First Conference on Hematopoiesis, Milan (1971), pp. 7-20.
14. M. Bessis, in: W. J. Williams (ed.), Hematology, 3rd Edition, New York (1986), pp. 257-279.
15. E. Robbins, R. Marcus, and N. Gonatas, Cell. Biol., **21**, 49 (1964).
16. J. Yoffey and P. Yaffe, J. Reticuloend. Soc., **28**, 37 (1980).