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HAEMOPOIETIC SYSTEM OF THE ANURANS: THE ROLE OF BONE MARROW AND LIVER

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Haemopoietic System of the Anurans: the Role of Bone Marrow and Liver. Akulenko N. M. — The haemopoietic activity of the frog, *Pelophylax ridibundus* was investigated during the year. Liver and bone marrow myelograms were examined in the different seasons using the special indexes and coefficients. It was shown the presence of the erythroid and granulocytic differentiation during the year in the both organs. In the bone marrow is changing the total number of the non-diferentiated haemopoietic cells, but ratio between erythroid and granulocytic progenitors is stabile. The haemopoietic activity of the liver has more prominent season variation in comparison with the marrow, but their total significance is comparable. The erythrocytic differentiation is more prominent during the summer and autumn, but granulocytic one took place in the spring and summer.

Key words: haemopoiesis, anuran.

Гемопоэтическая система бесхвостых амфибий: роль костного мозга и печени. Акуленко Н. М. — Гемопоэтическую активность костного мозга и печени озерной лягушки (*Pelophylax ridibundus*) исследовали в течение года. С помощью специальных коэффициентов и показателей анализировались миелограммы костного мозга и печени, определенные в различные сезоны. Показано наличие в обоих органах дифференцировки эритроидного и миелоидного ростков в течение года. В костном мозге изменяется общее количество недифференцированных предшественников кроветворения, но соотношение эритроидных и гранулоцитарных предшественников остается стабильным. Гемопоэтическая активность печени имеет более выраженные сезонные колебания, хотя её общее значение для гемопоэза сравнимо с костным мозгом. Дифференцировка эритроцитов более выражена летом и осенью, а дифференцировка гранулоцитов — весной и летом.

Ключевые слова: гемопоэз, бесхвостые амфибии.

Introduction

Topographical proximity of the hematopoietic organs with supporting tissue (bone or cartilage) is a general evolutionary tendency for different groups of vertebrates (Akulenko, 2008 a). Hematopoietic loci occurred several times in the evolution on the various parts of the skeleton (notochord, the skull, bonesof the limbs, bony skeleton as a whole). In different evolutionary branches stromal basis for blood-forming organs, which are topographically associated with the skeleton, were different types of connective tissue membranes (the periosteum, the endosteum, the meninges, the nerve trunk and others). This diversity shows that in the hematopoiesis stimulation the interaction with the bone or cartilage tissue played the leading role. Indeed, observation of the mammalian haemopoesis has produced overwhelming evidence that exposure to bone tissue, in particular osteoblasts, is an important factor of its regulation. According to modern views, there are "niches" on the bone surface in which the conditions are created for maintaining the proliferation of the nondifferentiated hematopoietic precursors and their subsequent differentiation (Forsberg, Smith-Berdan, 2009). Thus, the contact between the bone, cartilage and hematopoietic tissues are functionally validated.

However, in anamnia vertebrates hematopoietic foci that are associated with bone or cartilage tissue constitute only a portion of the hematopoietic system. Hematopoiesis is found in liver, kidney, spleen, gonads, intestinal submucosa, pericardium, etc. (Akulenko, 2008 a). Studies on amphibians have shown that the bloodstream under certain conditions may function as a fully-fledged department of the hematopoietic

system (Maslow, Tavrovsky, 1993; Akulenko, 2011). However, peculiarity of the heamatopoiesis outside the bone marrow cavity in adult amphibians representatives have not been studied enough. In particular, there are indications that hematopoiesis in the liver of anurans exists in adult animals (Spornitz, 1975; Thomas, Maclean, 1975; Koya et al., 1999; Nogawa-Kosaka et al., 2011) while there is a research denying this argument (de Abreu et al., 2009).

The aim of the present study is to analyze, using previously developed methods (Akulenko, 2009, 2011), the characteristic features of the main lines blood cells differentiation in the bone marrow and liver of anurans representative, lake frog, to compare them with each other and with the peculiarities of blood cells differentiation in the bloodstream, which have been described in previous work (Akulenko, 2011). Material was collected throughout the year and it allowed us to estimate the activity of bone marrow and liver in various phases of the life cycle when different germ cell differentiation is activated, or, conversely, suppressed.

Material and methods

The studies were conducted on 45 male lake frogs (*Pelophylax ridibundus* (Pallas, 1771), which were taken (each 2 animals with pauses from 2 weeks to 1 month) for 2 years. Sexually mature (8–10 cm, 45–50 g), frogs were taken from unpolluted sites. The animals were grouped in three samples of 15 specimens depending on the time of the intake: spring (April, May), summer, autumn and winter (time of hibernation, from September to March inclusive). Myelograms were counted on smears of bone marrow and smears of liver, stained by Pappenheim. Simultaneously, the histological preparations of bone marrow and liver were stained with hematoxylin-eosin. For the analysis of erythrocytic and granulocytic differentiation activity, we used average and error (M, m), coefficients of variation (CV) and some special parameters (total number of cells of granulocytic series, the immature myeloid cells, immature erythroid cells, etc.) (see tables 1, 3). The following relationships were defined: erythroblasts: basophilic normoblasts, erythroblasts: polychromatic normoblasts, polychromatic normoblasts: basophilic, and myeloid cells immature: mature. For the latter figure the total number of immature myeloid cells in the animal (myeloblasts + myelocytes neutrophilic + metamyelocytes neutrophilic + myelocytes eosinophilic + metamyelocytes eosinophilic) was divided by the total number of mature myeloid cells (stab and segmented neutrophils and eosinophils). Special rates were calculated for each computed myelogram, and then we determined their average value for the sampling error, and the reliability of differences between samples. Calculations were performed in Microsoft Excel according to our methodology (Akulenko, 2009). Determination of significance of differences between samples and the reliability of correlation was performed by standard methods using t-test.

Results and discussion

When we compare the bone marrow myelograms for the different seasons, the result was largely unexpected. It turned out that despite the high individual variability of the animals from natural populations, the mean values for each type of bone marrow cells are stable. Almost all of the data for different seasons (tables 1, 2, 3) are of the same size, taking into account the statistical error.

This is surprising, because during the year noticeable changes occur in the bone marrow of the anurans. During the wintering the number of hematopoietic cells in the bone marrow is strongly reduced (see Khamidov et al., 1978, and more). Much of the marrow cavity is filled with fat cells. At all seasons, a layer of differentiated blood cells is located along the inner surface of the bone, adhering to the bone and cartilage, which clearly confirms the theory of "niche". In winter, this layer becomes very thin (due to the small number of myeloid tissue in the bone marrow cavity during the wintering, we were able to calculate the myelogram only for 4 animals). In addition, in the bloodstream of the lake frog, the differentiation of granulocytes is activated in the spring and lasts during summer, while erythroid hematopoiesis is activated in late summer and continues during wintering with less intensity (Akulenko, 2008, 2011). It would be logical to expect that the composition of the bone marrow reflects these changes. However, the percentage of different cell types in the bone marrow fluctuates around certain stable values and relations which are the same throughout the year or at least from April to August, when the animal has an active life (see tables 1, 2, 3). Where the differences between spring and summer really exist, they are significant. For example, the composition of bone marrow macrophages is updated in the spring (table 3, column "young macrophages", the difference between summer and spring is significant, $P < 0.05$). During the summer in the bone marrow increases the proportion of stab granulocytes

Table 1. Indicators of lake frog erythropoiesis in the myelogramms of the liver, peripheral blood and bone marrow in different seasons (in%) and the reliability of differences between them

Таблица 1. Показатели эритропоэза из миелограмм печени и костного мозга лягушки озерной в различные сезоны (в %) и достоверность различий между ними

Spring							
Indicators	Liver			Bone marrow			Reliabil. differences oven: bone marrow: liver
	M	m	CV	M	m	CV	
Immature erythroid cells (total)	11	2.7	97	18	2.3	50	$P < 0.05$
Erythroblasts	1.7	0.3	77	2.6	0.3	49	$P < 0.1$
Basophilic normoblasts	2.5	0.8	123	5.8	0.8	57	$P < 0.05$
Polychromatic normoblasts	6.4	1.9	117	9.4	1.4	57	
Erythroblasts: basophilic normoblasts	1.1	0.4	119	0.6	0.1	62	
Erythroblasts: polychromatic normoblasts	0.5	0.1	111	0.4	0.1	89	
Basophilic normoblasts: polychromatic normoblasts	0.4	0.1	88	0.6	0.1	46	$P < 0.05$

Summer							
Indicators	Liver			Reliabil. differences oven liver spring: summer	Bone marrow		
	M	m	CV		M	m	CV
Immature erythroid cells (total)	22	4.6	71	$P < 0.05$	19	3.6	66
Erythroblasts	2.7	0.5	58		3.7	0.6	59
Basophilic normoblasts	6.2	1.3	73	$P < 0.05$	5.7	0.8	48
Polychromatic normoblasts	15	3.1	69	$P < 0.05$	11	2.2	72
Erythroblasts: basophilic normoblasts	0.5	0.1	75		0.6	0.1	55
Erythroblasts: polychromatic normoblasts	0.5	0.3	232		0.6	0.2	83
Basophilic normoblasts: polychromatic normoblasts	0.4	0.1	76		1.0	0.4	116

Autumn — Winter							
Indicators	Liver			Reliabil. differences oven liver autumn: summer	Bone marrow		
	M	m	CV		M	m	CV
Immature erythroid cells (total)	12	3.6	104	$P < 0.1$	12	4.7	102
Erythroblasts	2.2	0.5	79		2.5	1.4	148
Basophilic normoblasts	2.4	0.8	109	$P < 0.05$	3.2	1.2	100
Polychromatic normoblasts	6.9	2.8	134	$P < 0.1$	6.5	2.7	108
Erythroblasts: basophilic normoblasts	0.7	0.1	47		0.5	0.2	80
Erythroblasts: polychromatic normoblasts	0.5	0.1	66		0.4	0.2	100
Basophilic normoblasts: polychromatic normoblasts	0.5	0.3	199		0.4	0.2	95

and decreases that of segmented, indicating a more rapid exit of mature granulocytes from the bone marrow into the circulation ($P < 0.05$). However, these are small, easily explainable differences, which only emphasize the overall stability of the picture. If we consider the summary measures: the total number of erythroid (immature) cells, myeloid cells (belonging to the 2nd granulocytic lines of differentiation), the ratio of immature and mature cells in each line, etc., then the average values of these indices during the year are even more stable than the average values of myelogram.

In the liver during the year, fluctuations are detected in the number of individual cell types in the myelogram. Statistically significant changes in the number of erythroblasts and normoblasts (table 1) confirm the conclusion that the differentiation of red blood cells are found in the liver during the whole year, but in summer its level was significantly higher (Akulenko, 2008). At the same time, significant differences between

Table 2. Indicators of lake frog granulocyte differentiation myelogramms liver, peripheral blood and bone marrow a in different seasons (in%) and the reliability of differences between them

Таблица 2. Показатели дифференцировки гранулоцитов из миелограмм печени и костного мозга лягушки озерной в различные сезоны (в %) и достоверность различий между ними

Spring							
Indicators	Liver			Bone marrow			Reliabil. differences oven: b. m: liver
	M	m	CV	M	m	CV	
Myeloid cells (total)	25	2.5	97	34	5.6	63	
Myeloblasts	1.6	0.3	77	3.1	0.8	105	
Neutrophilic myelocytes	1.8	0.3	63	4.7	1.4	116	P < 0.05
Eosinophilic myelocytes	1.2	0.3	84	3.6	1	108	P < 0.05
Neutrophilic metamyelocytes	1.9	0.5	99	5.2	1.5	108	P < 0.05
Eosinophilic metamyelocytes	1.6	0.5	107	4.8	0.9	69	P < 0.001
Neutrophils, stab	5	1	80	4	0.9	92	
Eosinophils stab	1.8	0.5	117	2.7	0.4	63	
Segmented neutrophils	5.8	1.2	81	3.6	1.3	136	
Segmented eosinophils	4	0.7	69	2.6	0.5	69	
Basophils	5.3	1.5	117	0.4	0.2	250	P < 0.001
Immature myeloid cells	8.2	1.0	49	21	4.9	89	P < 0.05
Immature myeloid cells: mature	0.7	0.1	68	2.1	0.5	93	P < 0.05
All stab	6.7	1	56	6.7	1	59	
All segmented	9.8	1.8	70	6.1	1.4	87	

Summer								
Indicators	Liver			Reliabil. differences oven liver spring: summer	Bone marrow			Reliabil. differences oven: b. m: liver
	M	m	CV		M	m	CV	
Myeloid cells (total)	26	3.3	44		36	5	48	
Myeloblasts	0.6	0.2	98	P < 0.05	2.6	0.3	41	P < 0.001
Neutrophilic myelocytes	1.2	0.3	83		4.4	0.7	57	P < 0.001
Eosinophilic myelocytes	1.2	0.3	108		3.3	0.4	46	P < 0.001
Neutrophilic metamyelocytes	1.4	0.4	107		5.9	1.1	64	P < 0.01
Eosinophilic metamyelocytes	1.2	0.2	119		6.7	0.9	48	P < 0.001
Neutrophils, stab	8.3	2.1	87		8.2	1.8	78	
Eosinophils stab	3.5	0.8	78	P < 0,1	4.8	1	75	
Segmented neutrophils	6.3	1.6	110		1.2	0.3	87	P < 0.01
Segmented eosinophils	6.3	1.2	86		1.4	0.4	93	P < 0.001
Basophils	7.5	1.4	80	P < 0,1	0.3	0.2		P < 0.001
Immature myeloid cells	5.5	1	76	P < 0,1	21	2.8	46	P < 0.001
Immature myeloid cells: mature	0.3	0.1	89	P < 0,05	1.6	0.2	52	P < 0.001
All stab	11	2	64	P < 0,1	12	2.7	78	
All segmented	12	2.9	100		2.3	0.4	53	P < 0.01

Autumn — Winter								
Indicators	Liver			Reliabil. differences oven liver autumn: spring	Reliabil. differences oven liver autumn: summer	Bone marrow		
	M	m	CV			M	m	CV
Myeloid cells (total)	24	2.9	39			32	7.4	61
Myeloblasts	0.6	0.2	125	P < 0.01		2.5	0.7	70
Neutrophilic myelocytes	1.2	0.3	98			4.1	1.1	68
Eosinophilic myelocytes	0.6	0.2	125	P < 0.05	P < 0.05	2.8	0.9	82
Neutrophilic metamyelocytes	1.3	0.4	108			4.8	1.4	76
Eosinophilic metamyelocytes	1.1	0.3	104			3.4	1.3	105

Table 2.
Окончание табл. 2.

Autumn — Winter								
Indicators	Liver			Reliabil. differences oven liver autumn: spring	Reliabil. differences oven liver autumn: summer	Bone marrow		
	M	m	CV			M	m	CV
Neutrophils, stab	7.7	1.4	61			5.3	1.4	69
Eosinophils stab	2.9	0.9	99			2.6	0.6	67
Segmented neutrophils	6.3	0.8	42			4.8	1.6	86
Segmented eosinophils	2.7	0.7	89		$P < 0.01$	2.2	1.4	169
Basophils	4.8	1.7	118			0.8	0.2	147
Immature myeloid cells	4.8	1	68	$P < 0.05$		18	4.7	71
Immature myeloid cells: mature	0.3	0	59	$P < 0.01$		1.1	0.4	89
All stab	11	1.8	56	$P < 0.05$		7.8	1.8	62
All segmented	9	1.1	39			7	2.7	103

bone marrow and liver indices, which are related to erythropoiesis are absent in the summer sample myelogram. Moreover, in the summer and autumn, there are no significant differences in parameters of erythropoiesis between bone marrow, liver and the circulating blood. The only exception is the ratio "erythroblasts: basophilic normoblasts", "erythroblasts: polychromatic normoblasts" and "basophilic normoblasts: polychrome". In the bone marrow and liver during the spring and summer these ratios are several times higher than in the peripheral blood, and differences are significant. (Data on the peripheral blood are shown in a previous publication (Akulenko, 2011). Thus, we can assume that the liver of anurans along with their bone marrow may be a donor of the erythroblasts and basophilic normoblasts, which continue to differentiation and proliferation in the vascular bed. This conclusion coincides with the most recent data in literature (Thomas, Maclean, 1975; Chegini et al., 1979; Maslova, Tavrovsky, 1993; Koya et al., 1999; Nogawa-Kosaka et al., 2011). Comparing the coefficients of variation, we can also conclude that the individual variability in the intensity of erythropoiesis in the liver in the spring and summer is more prominent than in the bone marrow, although in the summer this difference is weak. During the wintering individual variability of erythropoiesis in all loci, including peripheral blood, is expressed most strongly.

All indicators of myelopoiesis (i. e., differentiation of granulocytes) in the liver during the year were significantly lower than in the bone marrow (table 2). However, the number of myeloblasts and myelocytes in the liver was significantly higher than in the peripheral blood during summer ($P < 0.001$) and autumn ($P < 0.01$). In spring the differences are less reliable: $P < 0.05$ and $P < 0.1$ for various parameters. During the year, the largest number of immature myeloid cells in the liver is found in spring. In summer these indicators are lower, but not always this reduction is significant. When we consider the coefficients of variation, it appears that the most stable differentiation of granulocytes is found in summer in the bone marrow, followed by the liver in spring. Judging from the data in table 2 and 3, in spring the liver seems to play a pivotal role in recovery from hibernation of non-specific defenses mechanisms which are mediated by granulocytes and monocytes-macrophages. In summer the liver passes this role to bone marrow. Individual variability in the number of early progenitor granulocytopoiesis in the liver and bone marrow is significantly lower than in the bloodstream. In the bloodstream coefficients of variation of the number of myeloblasts and myelocytes in different seasons vary between 125–360 % (Akulenko, 2011); in the liver and bone marrow, these coefficients do not exceed 125 % (table 2). From this fact we can

Table 3. Indicators related to the immunocompetent cells of the myelogramms liver and bone marrow frog lake in different seasons (in%) and accuracy differences between them

Таблица 3. Показатели, относящиеся к иммунокомпетентным клеткам из миелограмм печени и костного мозга лягушки озерной в различные сезоны (в %) и достоверность различий между ними

Spring							
Indicators	Liver			Bone marrow			Reliabil. differences oven: b. m: liver
	M	m	CV	M	m	CV	
Monocytes	1.5	0.4	91	0.5	0.2	159	P < 0,05
Young macrophages	3.4	0.9	105	0.5	0.2	124	P < 0,01
Macrophages	5.8	1.2	81	1.8	0.4	83	P < 0,01
lymphoblasts	4.8	0.7	58	3.8	0.4	42	
Lymphocytes	41	3.2	30	37	5.2	54	
plasmocytes	1.3	0.3	89	1,1	0.2	66	
Non-differentiated blasts	1.8	0.6	123	1,6	0.3	75	
Mitoses	0.6	0.2	134	1	0.3	99	
All blasts	10	1.4	55	11	1.3	46	

Summer								
Indicators	Liver			Reliabil. differences oven liver spring: summer	Bone marrow			Reliabil. differences oven: b. m: liver
	M	m	CV		M	m	CV	
Monocytes	0.7	0.1	73	P < 0.05	0.3	0.1	93	P < 0.05
Young macrophages	0.8	0.3	125	P < 0.05	0.1	0.1	161	P < 0.01
Macrophages	4.4	1.1	85		1	0.3	234	P < 0.001
lymphoblasts	1.5	0.4	95	P < 0.001	2.4	0.5	97	
Lymphocytes	30	2.6	29	P < 0.05	31	4.3	73	
plasmocytes	0,4	0.2	200	P < 0.05	1.9	0.4	48	P < 0.05
Non-differentiated blasts	1.9	0.6	101		2.5	0.6	82	
Mitoses	0.4	0.2	150		1.5	0.3	85	P < 0.001
All blasts	6.5	1.1	60		10	1.5	48	P < 0.05

Autumn–Winter									
Indicators	Liver			Reliabil. differences oven liver autumn: spring	Reliabil. differences oven liver autumn: summer	Bone marrow			Reliabil. differences oven: b. m: liver
	M	m	CV			M	m	CV	
Monocytes	0.5	0.2	149	P < 0.05		1	0.4	101	
Young macrophages	2.3	0.7	109		P < 0.05	0	0		P < 0.01
Macrophages	6	1.6	87			5	2.1	112	
Lymphoblasts	2.5	0.7	96	P < 0.05		1.8	0.7	106	
Lymphocytes	45	3.3	24		P < 0.001	25	7.8	85	P < 0.05
Plasmocytes	1.2	0.5	152			0.2	0.1	224	P < 0.05
Non-differentiated blasts	1.4	0.5	127			2	0.8	108	
Mitoses	0.2	0.1	173	1.9 P < 0.1		0.1	0.1	224	
All blasts	6.7	1.4	69	1.8 P < 0.1		8.8	2		

conclude that the differentiation of granulocytes in the bone marrow and liver throughout the year is normal for the lake frog physiological process, while the bloodstream joins in only under the conditions of high demand in these cells.

These data show that the liver in anurans plays a very important role in hematopoiesis, at least comparable to the role of bone marrow especially given that that the total volume of bone marrow in anurans is ten times smaller than the volume of the liver. This ratio of bone marrow hematopoiesis and hematopoiesis in the liver can hardly be considered a special case unique only to the lake frog. Oxygen demand and the need in the cellular defense mechanisms in all anurans are similar, but the relative value of bone marrow cavity of a lake frog is even higher compared to other anurans.

From this point of view it looks somewhat odd that the majority of authors who wrote about the hematopoiesis in anurans tend to underestimate the role of the liver (Khamidov et al., 1978). The possible explanation may be in the fact that in the liver of anurans myeloid tissue does not form morphologically-looking inclusions. (Except for subcapsular layer of myeloid tissue, that does not exist in all species). In the lake frog's liver differentiating granulocytes are scattered among the hepatocytes or in melanomacrophagal aggregations. They form small groups of several cells. Maturing erythroid cells are found in the sinusoids of the liver, but there they are "diluted" by a large number of mature red blood cells. Thus, the process of hematopoiesis in the liver of anurans is difficult to identify. Only the modern methods such as immunofluorescent microscopy (Nogawa-Kosaka et al., 2011) or the method of cell culture (Koya et al., 2009) allow finding the undifferentiated progenitor cells of the hematopoiesis in the liver. Our method of calculations on the smears of the liver also reveals immature progenitor cells and, moreover, makes it possible to quantitatively analyze and compare the processes of differentiation.

The second difficulty is that the composition of myelogram in the liver is more susceptible to seasonal fluctuations than in the bone marrow. If the animals are captured in spring when erythropoiesis is minimal, it may be perfectly correct conclusion that this process is limited by the bone marrow. However, it will be correct only for spring. Upon activation of erythropoiesis, it involves the liver, spleen and bloodstream (Maslow, Tavrovsky, 1993; Akulenko, 2008, 2011). Thus, the liver occupies an intermediate position between the bone marrow and bloodstream. Seasonal variations in the differentiation of hematopoietic cells of various lines in it are more pronounced than in the bone marrow. On the other hand, seasonal and individual variations of these processes are expressed in the circulation much more distinctly than in the liver (Akulenko, 2011).

Conclusion

The role of the lake frog's liver in the process of hematopoiesis is quite comparable to that of bone marrow. In it there is differentiation of the red blood cells, granulocytes, certain subpopulations of lymphocytes. However, the processes of hematopoiesis in the liver are more susceptible to seasonal fluctuations than in the bone marrow.

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