# ON THE CELLULAR AUTOIMMUNE MECHANISM FOR ELIMINATING ERYTHROCYTES NORMALLY AND UNDER EXTREME INFLUENCES

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16. Abstract This paper demonstrates the presence of an autoimmune cellular mechanism for destroying erythrocytes on the basis of results of experiments in vivo. In the blood and the organs this mechanism is made up of a population of immunocompetent killer-lymphocytes which originates in the bone marrow and the thymus, and which is manifested in the local hemolysis effect.

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# ON THE CELLULAR AUTOIMMUNE MECHANISM FOR ELIMINATING ERYTHROCYTES NORMALLY AND UNDER EXTREME INFLUENCES

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The mechanisms for the physiological destruction of erythrocytes /16* normally, as well as under extreme influences (hypoxia, loss of blood, repeated cobalt injections) which cause accelerated erythropoiesis, have been inadequately studied. Ya.G. Uzhanskiy [8] proposed an hypothesis about the immune nature of the mechanism of erythropoiesis. However, the role of humoral immunity factors (autoantibodies) in the mechanism for eliminating erythrocytes was not confirmed experimentally [10, 13, 16]. We have described earlier the phenomenon of accelerated plaque formation by means of autoerythrocytes during erythropoiesis' activation [2, 3, 7], forming a population of immunocompetent cell-killers which originates in the bone marrow and the thymus [5]. The normal presence of auto-plaque-forming cells (APFCs) in the blood and the organs, and the consistent increase in their number under stressful influences, which is accompanied by accelerated hemolysis [9], allowed the conclusion that the APFCs are responsible for the identification and elimination of erythrocytes, with variable antigenic determinants, which appear as a result of physiological aging or accelerated aging which results from amplified functional activity of the red-blood system. The fact that the antigenic structure of the surface proteins of the erythrocyte's membrane changes as a result of aging is indicated by data about their loss of a superficial electric charge, changes in the conformational properties of the superficial proteins of the membrane [15], and their denaturation [12]. In experiments in vitro, direct evidence of change in the antigenic properties of metabolism-dependent superficial antigenic cells under stressful influences [17, 18], and also different antigenic qualities of erythrocytes /17

*Numbers in the margin indicate pagination in the foreign text.

of different age compositions [11, 14], was obtained.

In order to verify the action of APFCs in the mechanism for erythrocytes' physiological elimination, the present work examines, in experiments in vivo: 1.) The dependence of the erythrocytes' stability on the concentration of APFCs in the surrounding blood; 2.) The ability to develop post-transfusion polycythemia against the background of the cellular autoimmune reaction's excitation; 3.) The magnitude of the hemolysis effected by the APFCs in contrast to the hemolysis calculated by the average duration of the erythrocyte's life.

Method

Mice of the CBA and $F_1$ (CBA x $C_{57}Bl_6$) strains, females two to four months old, obtained from the incubator of the USSR Academy of Medical Sciences' "Stolbovaya" animal laboratories, were used.

The dependence of the circulating erythrocytes' stability on the concentration of APFCs in the blood was investigated in an experimental model of the peaks of APFCs obtained in the peripheric blood, which had various temporal characteristics depending on the level of hypoxic effect [4]. Two experimental series of 70 mice of the $F_1$(CBA x $C_{57}Bl_6$) strain were established.

In the first series (I), the animals were subjected to constant barometric hypoxia at 0.75 atmospheres for thirteen days; in the second series (II), at 0.5 atmospheres for six days. The chamber was opened once every 24 hours for ten to fifteen minutes in order to take care of the animals. Studies were made three and ten hours after the hypoxic effect, on the first, third, and sixth days, and, under hypoxic conditions at 0.75 atmospheres, also on the tenth and thirteenth days. Each group contained five mice. In order to count the quantity of erythrocytes destroyed by the APFCs, experiments were conducted in vitro on the CBA-strain mice. The effect of the excited reaction of the autoimmune plaque formation on the development of polycythemia was studied in the mice of the

$F_1$(CBA x $C_{57}Bl_6$) strain, who twice underwent transfusion of 0.9 milliliters of a ninety percent suspension of syngenic erythrocytes. The interval between the injections was 24 hours. Seven hours after the second transfusion, 0.5 milliliters of a fifty percent suspension of xenogenic (rabbit) erythrocytes was introduced into the animals of the experimental group. The quantity of APFCs in the blood and the organs was studied on the first, second, and third days after the second transfusion. Hematocrit was determined, according to the standard method, in the capillaries after centrifuging at 3000 RPM for thirty minutes.

APFCs were detected with a modification [4] of N.N. Klemparskii's method. The distribution of erythrocytes by stability was investigated using the method of acid erythrocytes [7]. Analysis of the distribution was conducted by the average erythrogram obtained from four to five mice as a result of a linear transformation of each erythrogram with their subsequent combination on a point which corresponds to the time of 50 percent of the hemolysis of the erythrocytes (t50).

Results and Discussion

An analysis of the kinetic formation of the APFC population during hypoxic influence in 0.75 and 0.5 atmospheres (figure 1) showed its dependence on the degree of the influence: the high level of hypoxia (under 0.5 atmospheres) guarantees the formation of a maximum of APFCs at the place of localization of the antigen's basic mass (in the peripheric blood), and an earlier time -- after three hours. At that time, as during the hypoxic effect in 0.75 atmospheres, the maximum of the relative quantity of APFCs was noted on the sixth day. It should be mentioned that an unnoticeable, though significant, increase in the quantity of APFCs in the blood also occurs after three hours under the effect of hypoxia in 0.75 atmospheres, while the quantity of APFCs decreases at that time in the bone marrow and the spleen (table 1).

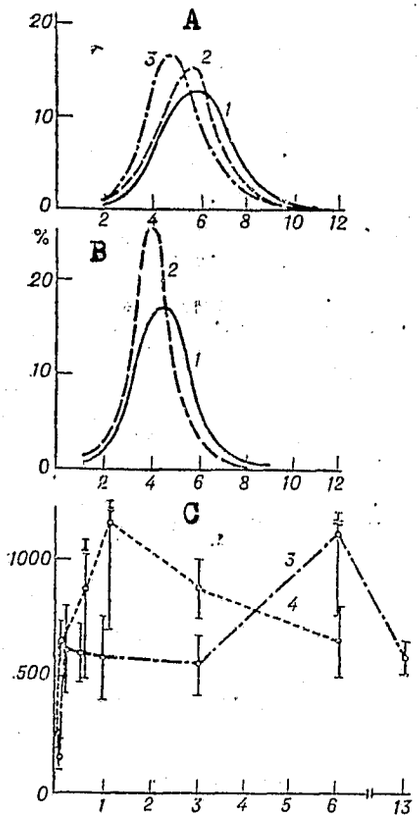


Figure 1

Erythrocytes' stability (A and B), and the APFC concentration (C), in the peripheric blood under various degrees of hypoxic influence.

For A, under 0.75 atmospheres, and B, under 0.5 atmospheres:
1 - background distribution by stability;
2 - 3 hours after the hypoxic influence;
3 - 6 days after the hypoxic influence.

For C:
3 - APFC concentration in the peripheric blood under 0.75 atmospheres;
4 - APFC concentration in the peripheric blood under 0.5 atmospheres.

Along the abcissae:
- time in minutes (for A and B)
- time in days after the hypoxic influence (for C)

Figure 1 (continued)

Along the ordinates:
- erythrocytes' stability (percent) (A and B);
- number of APFC in one cubic millimeter (for C).

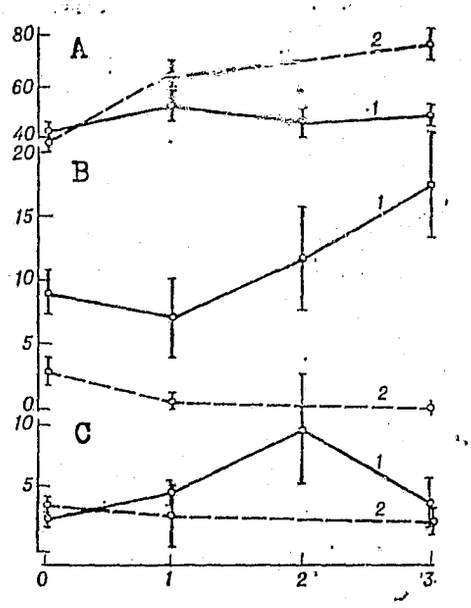


Figure 2

The dynamics of hematocrit (A), the APFC content in the peripheric blood (B), and the spleen (C), in mice of the $F_1$(CBA x $C_{57}Bl_6$) strain, against a background of a twice-repeated transfusion (control) and with an analogous transfusion with the consequence of introducing an immune dose of rabbit erythrocytes.

1 - experiment; 2 - control

Along the abcissae: the time (in days) after the introduction of the rabbit erythrocytes;
along the ordinates: percent hematocrit (for A) and percent APFCs (for B and C).

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The doubling time for the number of APFCs in the peripheric blood is less than three hours. This may be related to an addition to the APFC pool by means of the recruitment and migration mechanisms. This conclusion is indirectly indicated by the decline in the relative

Table 1

APFCs (percent) IN THE PERIPHERIC BLOOD AND IN THE ORGANS UNDER DIFFERENT LEVELS OF HYPOXIC INFLUENCE IN MICE OF THE $F_1$(CBA x $C_{57}Bl_6$) STRAIN ($\underline{M} \pm \underline{m}$)

| 1 Уровень гипоксии | 2 Органы и ткани | 3 Фон | 4 Время после гипоксического воздействия | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 5 3 ч | 6 10 ч | 7 24 ч | 8 3 сут | 9 6 сут | 10 10 сут | 11 13 сут |
| 12 0,75 атм | 14 Кровь | 3,8±0,9 | 25,6±6,8* | 12,3±1,7*** | 14,8±5,4 | 17,4±2,7*** | 36,5±4,7*** | 26,4±3,9*** | 15,8±2,8** |
| | Костный мозг 15 | 16,22±3,8 | 7,05±0,98* | 10,7±2,2 | 16,5±3,4 | 18,3±5,5 | 34,2±8,7 | 35,9±7,9 | 24,5±5,7 |
| | 16 Селезенка | 8,3±2,4 | 4,8±0,7 | 8,0±2,6 | 8,9±2,1 | 9,7±2,3 | 12,2±4,9 | 13,2±2,5 | 15±3,9 |
| 13 0,5 атм | Кровь 17 | 8,2±1,7 | 18,6±2,5** | 19±5,2* | 15,7±2,6* | 12,6±2 | 9,4±3 | | |
| | 18 Костный мозг | 14,6±2,8 | 14,9±5,4 | 22,6±5,8 | 20,9±2,6 | 13,1±3,1 | 15,7±2 | | |
| | Селезенка 19 | 6,5±1,4 | 9±2,1 | 11,7±1,3* | 11,6±0,8* | 5,3±2,3 | 6,3±0,4 | | |

* $P<0,05$.
** $P<0,01$.
*** $P<0,001$.

Note: P was calculated in relation to the background.

[Commas in tabulated material are equivalent to decimal points.]

Key:

1. level of hypoxia
2. organs and tissues
3. background
4. time after the hypoxic influence
5. 3 hours
6. 10 hours
7. 24 hours
8. 3 days
9. 6 days
10. 10 days
11. 13 days
12. 0.75 atmospheres
13. 0.5 atmospheres
14. blood
15. bone marrow
16. spleen
17. blood
18. bone marrow
19. spleen

quantity of APFCs in the organs after three hours under 0.75 atmospheres, or their constant level at the same time under 0.5 atmospheres. During hypoxia at 0.75 atmospheres, the consistent growth in the quantity of APFCs from the first through the sixth days indirectly indicates that, under low-level hypoxia, the addition to the APFC pool is caused by a process of proliferation. In the course of the experiment, the kinetics of the absolute quantity of the APFCs in the blood and the distribution of erythrocytes by stability were compared. Under the effect of hypoxia at 0.75 atmospheres, two leftward shifts in the erythrogram, which indicated hemolysis and a decline in the erythrocytes' resistance, were noted; /18 they corresponded to two peaks in the increase of the number of APFCs (see figure 1, A). Under the influence of hypoxia at 0.5 atmospheres, the earlier (after three hours) increase in the number of APFCs in the peripheric blood also corresponded to the first shift to the left in the erythrogram (see figure 1, B). In this manner, with only a sharp increase in the number of APFCs in the peripheric blood, a decrease in the stability of the erythrocytes and hemolysis were found. An investigation of the influence of excitation of the cellular autoimmune reaction by means of the introduction of an immunogenic dose of xenogenic erythrocytes after the usual twofold transfusion of syngenic erythrocytes, which specifically decreased the number of APFCs, showed a significant increase in the quantity of APFCs in the peripheric blood in the animals of the experimental group on the first, second, and third days, while polycythemia did not develop. In the animals of the control group, in the same time, polycythemia arose against the background of a significant decrease in the number of APFCs (figure 2).

The results of the investigation, indicating the dependence of the stability of circulating erythrocytes in vivo on the concentration of APFCs, and the disruption of polycythemia during the excitation of the cellular autoimmune reaction, as well as the data from the microscopic analysis about the presence inside the plate of shadows, erythrocyte fragments, and spinous erythrocytes -- the prehemolytic stage of the cell -- permit the conclusion to be drawn that one of

Table 2

MORPHOLOGY OF THE APFCs OF THE PERIPHERIC BLOOD AND SPLEEN

| 1 Виды клеток | 2 Диаметр, (мкм) | 3 Количество клеток | 4 Ядерно-плазменное отношение | 5 % от всех АБОК |
|---|---|---|---|---|
| 6 Препараты крови с клетками селезенки | | | | |
| 7 Малый лимфоцит | 4,5—6,5 | 186 | 2,6 | 74,4 |
| 8 Средний » | 6,5—10 | 21 | 2,3 | 8,4 |
| 9 Большой » | 10—18 | 43 | 0,65 | 17,2 |
| 10 Препараты крови | | | | |
| 7 Малый лимфоцит | 4,5—6,5 | 54 | 2,0 | 62,8 |
| 8 Средний » | 6,5—10 | 15 | 2,4 | 17,4 |
| 9 Большой » | 10—18 | 17 | 1,4 | 19,8 |

[Commas in tabulated material are equivalent to decimal points.]

Key:

1. type of cell
2. diameter (mkm)
3. quantity of cells
4. nucleo-plasmic relationship
5. percent of all APFCs
6. specimens of blood with cells of the spleen
7. small lymphocyte
8. medium "
9. large "
10. specimens of blood

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the basic means of destroying erythrocytes in the organism is the APFC population. /19

We attempted to quantitatively evaluate the hemolysis which occurs by means of the APFC population in the peripheric blood, and to compare it with the hemolysis calculated according to the average length of the erythrocytes' lives. When calculating the quantity of destroyed erythrocytes, it is necessary to know the volume occupied by the APFCs themselves, which is calculated according to the average diameter of the cell. In specially prepared specimens of the peripheric blood and spleen cells, fixed and stained according to Romanovskii-Hymes' method, the APFCs' morphology was studied. It should be noted that a significant part of the APFCs were lysed. 400 unlysed cells were measured (the diameter of the cell and the nucleus), and identified as lymphocytes of various sizes with a

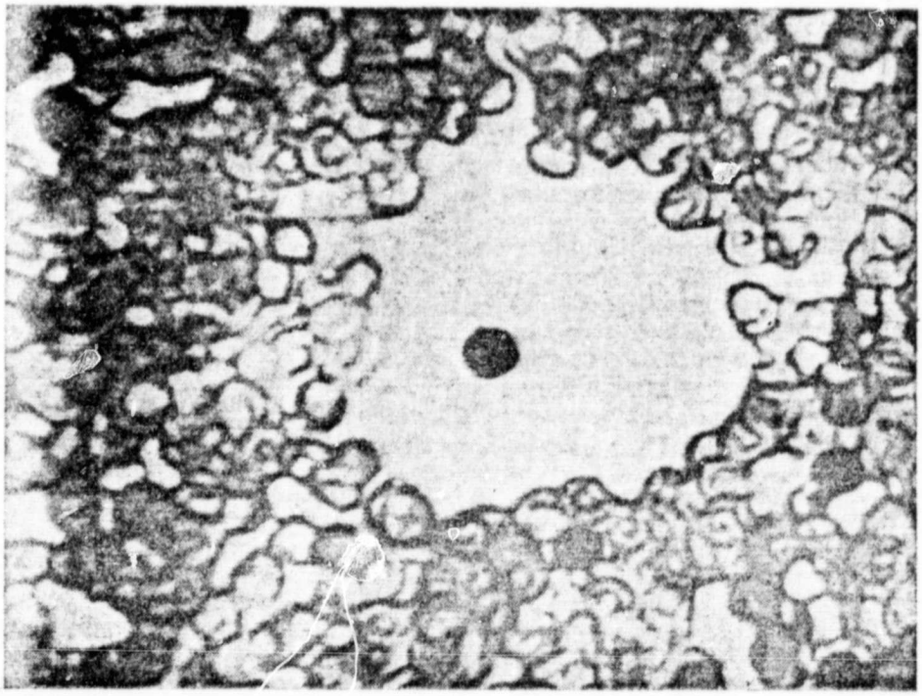

Figure 3

A fixed and stained specimen, showing the effect of plaque formation. There is a small lymphocyte in the center of the plaque. (60x objective, 10x eyepiece)

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nucleo-plasmic relationship (table 2, figure 3).

These data do not conclusively solve the problem of the morphology of the plaque-forming cells, since the lysed cells' morphology was not investigated, but they are evidence that lymphoid cells participate in the phenomenon of autoimmune plaque formation.

In experiments on intact and hypoxic mice of the CBA strain (four and six animals, respectively), the average quantity of erythrocytes which one APFC was capable of lysing in a definite amount of time was determined. In the specimens of peripheric blood and spleen cells, the quantity of plaques was counted and their diameter

measured dynamically immediately after ten minutes, one hour, five hours, and twenty to twenty-four hours. In all, 195 plaques were studied in intact mice and 419 in hypoxic ones.

The results of the investigation showed that the number of plaques and their average diameter in intact and hypoxic mice increased over the first five hours of incubation and did not change subsequently. The average diameter of the plaques after five hours was $18.7 \pm (2.6)(10^{3})/20$ and $20.8 \pm (1.2)(10^{-3})$ millimeters, respectively. In the specimen, the erythrocytes were so placed on the slide as to form a cellular layer, the thickness of which depended on the diameter of the chamber and on the quantity of erythrocytes utilized for the specimen's preparation. Obviously, not all the cells capable of realizing a hemolytic reaction manifested local hemolysis, but only those which were capable of lysing erythrocytes through the whole thickness of the layer. In the contrary case, the partial opacity of the erythrocytes in the layer would not have allowed identification as a plaque. The thickness of the cellular layer ($\underline{h}$) was calculated according to the formula $\frac{(V)(N)(Het)}{(S)(N_0)(100)}$, where $\underline{V}$ and $\underline{S}$ are the volume and area of the chamber; $\underline{Het}$ is the magnitude of hematocrit (percent) with a concentration of erythrocytes $\underline{N}_0$; and $\underline{N}$ is the concentration of erythrocytes in the specimen. Furthermore, it is assumed that the population density of the cells in the axial layer is equivalent to the population density of the cells during determination of hematocrit. Under our conditions, $\underline{S} = 3.24$ millimeters; $\underline{V} = 20$ cubic millimeters; $\underline{Het} = 40.7 \pm 0.6\%$; $\underline{N}_0 = 7.83 \pm (0.46)(10^{6})$ millimeters$^{-3}$; and $\underline{N} = (2)(10^{6})$ millimeters$^{-3}$. The thickness of the layer is then $(6.28)(10^{-3})$ millimeters. A plaque, with a diameter of $(18.7)(10^{-3})$ millimeters, is equivalent in shape to a cylinder formed during the lysing of the erythrocytes by the APFCs. The volume of this cylinder is

$$\pi(6.28)(10^{-3})\frac{((18.7)(10^{-3}))^2}{4} = (1725)(10^{-9}) \text{ mm}^3.$$

If the APFCs, with an average diameter of (7.1)(10.3) millimeters,

are deducted, then the destroyed erythrocytes would occupy a volume of $(1725-187)(10^{-9}) = (1538)(10^{-9})$ cubic millimeters. In such a volume,

$$\frac{(1538)(N)(100)(10^{-9})}{Het} = 30$$

erythrocytes are found. It follows that, on the average, one APFC is able to destroy about 30 erythrocytes in five hours. For hypoxic mice the hemolytic activity of the APFCs has turned out to be somewhat higher. /21

If the percentage of APFCs in the blood of intact mice of the given strain is 7.5 [7], and the quantity of leucocytes is $3460 \pm 190$ mm$^{-3}$, according to the data of the present experiment, then the quantity of APFCs in one millimeter is 261 cells. So, in one hour, these APFCs can destroy $(261)(30)/5 = (1.6)(10^3)$ erythrocytes in one cubic millimeter. With an average lifetime for erythrocytes in mice of forty days, and a concentration of erythrocytes of $(7.83)(10^6)$ cells in one cubic millimeter, the quantity of destroyed cells in one hour is $(7.83)(10^6/40)(24) = (8.2)(10^3)$ erythrocytes in one cubic millimeter.

In this manner, the hemolysis which is performed by the APFCs in the peripheric blood is about 19.5 percent of the hemolysis calculated by the erythrocytes' lifetime. The given calculation characterizes the minimum hemolysis performed by the APFCs. If the erythrocytes which are destroyed by those APFCs whose manifestations are limited by the sensitivity of the method, as well as the erythrocytes which are destroyed by the APFCs in the organs (in part, this mechanism may explain the ineffective erythropoiesis in the bone marrow, where the erythrocytes are destroyed without entering the channel), are considered, then it becomes obvious that the given hemolytic mechanism occupies a substantial place in the organism in both the steady and the stressed states of the sanguinary system.

Examining as a whole the ways of destroying erythrocytes in the organism and the causes which are conducive to them, one may propose the following chain of events: the cellular homeostasis of the

population of circulating erythrocytes is determined by their antigenic structure and by the controlling mechanisms of an immune nature, which eliminate erythrocytes in three stages. In the first stage, the erythrocytes with changed antigenic determinants are identified by specific receptors of the activated autoantigenic stimulus of the lymphocytes, which is manifested in vitro by an autoimmune plaque formation. As a result of the interaction of the specific structures of the APFC with the superficial autoantigenic determinants of the erythrocytes, the latter are broken into fragments or destroyed. This stage of destruction is completed by the second (intracellular) stage, when the erythrocytes, destroyed by the lymphocytes, or their fragments, are phagocytized, undergoing further intracellular digestion. If the phagocytic mechanisms and the cellular populations which take part in them are studied to a sufficient degree, then these questions in relation to the cell -- about the killers of the autologic erythrocytes (APFCs) -- which are only beginning to be studied, and their solution, will permit a closer approach to an understanding of the molecular mechanisms of cellular interaction which are conditioned by the processes of physiological aging and the destruction of erythrocytes.

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* The original of this citation says "vypusk 1, number 5". It seems likely that the first word (usually translated into English as "number") is a typographical error for "volume" -- translator.