A BLOOD CULTURE METHOD OF QUANTITATIVE ESTIMATION OF HEMOPOIETINS

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The hemopoietins, humoral erythropoietic substances found in blood and various tissue fluids during anoxia, have been studied extensively in recent years.

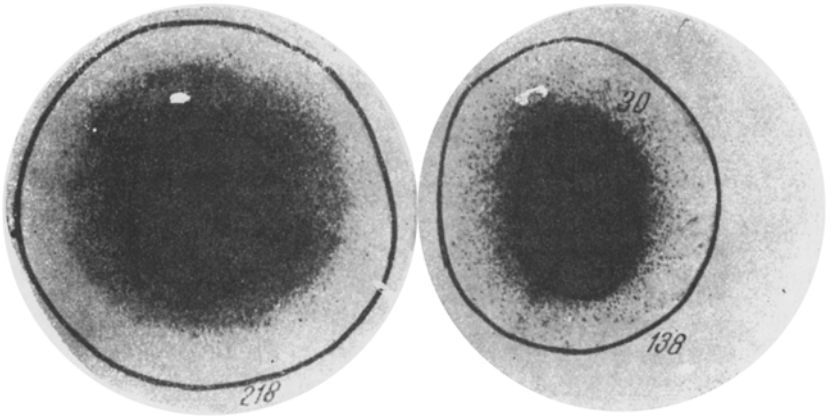
Different methods are used to detect hemopoietins. The indices of the peripheral blood, the morphological composition of the marrow, the rate of incorporation of radioactive iron into erythrocytes, the intermediate iron metabolism and other indices after injection of the test material into intact or anemized animals (rats, mice, rabbits, guinea pigs, dogs, etc.) may all be studied. All these methods are very laborious and time consuming and great care has to be taken with the animals, so that it is difficult to carry out the experiment on a large scale simultaneously.

In 1952 we suggested a biological method of quantitative estimation of hemopoietins, based on the ability of hemopoietically active substances to stimulate the migration of cells into cultures of leukocyte film [2].

The method is as follows. By means of a syringe, 10 ml of blood is taken from a donor's cubital vein or a cat's heart. The blood is transferred to two cold, waxed test tubes containing heparin (2-3 drops of 0.1% heparin solution to 5-6 ml blood), kept in a vessel with ice to prevent clotting of the blood. Ten minutes after collection (i.e., after cooling) of the blood, it is centrifuged for 10 minutes at 3000 rpm. The plasma is drawn off into a waxed test tube and placed on ice (this serves as a nutrient medium). The tubes with erythrocytes and leukocytes are incubated at 36.6-37° for 10 minutes for clotting to take place. The leukocyte film is then removed, and rinsed in a Petri dish with Ringer's solution until free from erythrocytes, and then transferred to another dish, where it is cut up into small pieces. The pieces of film are cultivated by the "hanging drop" method [8]. The material for testing is added to the nutrient medium in proportions of 1:1 during preparation of the cultures. If necessary it may be diluted with Ringer's solution, naturalized, and sterilized by microfiltration. Controls are set up, consisting of cultures of the same leukocyte film in which the plasma is mixed with an equal volume of Ringer's solution.

From 6 to 8 cultures are made with each specimen. The cultures are incubated at 36.6-37° for 6-7 hours. Then, by means of a drawing apparatus, pieces of film and the surrounding zone of migration from the living cultures are projected on to paper and measured with a planimeter. The index of migration of the cultures is calculated from the formula $O_2 - O_1 / O_1$, where O_2 is the circumference of the zone of migration, O_1 the circumference of the piece of film. The mean index of migration of 6-8 control cultures is taken as 100, and the differences in intensity of migration in the experimental cultures are expressed as a percentage of this figure, and conventionally regarded as an index of the hemopoietin content.

For example, measurement with the planimeter showed that the index of migration of an experimental culture, according to the above formula, was 218-33/33 = 5.6, and the index of migration of the control culture was 138-30/30 = 3.6 (see Fig. 1). Let us assume that after measurement of 6-8 experimental cultures, the mean index of migration is 5.4, and the mean index of migration of the control cultures is 3.5. The ratio between the migration of the experimental and control cultures will thus be 5.4:3.5, or 154%, and the result can be expressed as +54. If the index of migration of the experimental cultures is less than that of the controls, a negative result is obtained. If, for example, the mean index of 6-8 experimental cultures is 2.8, the ratio of the migration of the experimental cultures will be 2.8:3.5, or 80%, and the result will be -20.



a b

Cultures of leukocyte film after cultivation for 7 hours. a) Experiment; b) control.

In the preparation of the cultures the rules of asepsis are observed, and a sterile vessel is used. Attention must be drawn to the following point. The leukocytes are not always uniformly distributed in the film, and in some films or in some parts of a film leukocytes may be absent. It is therefore most important, when setting up the cultures, to use comparable pieces of film. On this depends the accuracy of the method. With a proper choice of film, the probable mean error will not exceed ±5.8, but otherwise it may be considerably increased. The optimal concentration of test material for the blood cultures must also be ascertained. For example, gastric juice and saliva should be diluted 8-10 times. Plasma or serum may be added to the nutrient medium without dilution.

If human film is used, an equal volume of rabbit's plasma must be added to its plasma (to prevent dilution). Blood from which the plasma is obtained is taken from a rabbit's heart, and heparin is added to the tube.

The choice of leukocyte film in preference to marrow as inoculum is determined by the fact that it contains more mature granulocytes. These cells are more mobile and migrate more rapidly from the piece of film than the young myeloid cells, and the difference between the zones of migration in the experimental and control cultures is therefore more obvious. Lymphocytes also migrate more slowly than granulocytes, so that it is preferable to use a film containing a larger number of granulocytes. Leukocyte film from dogs' blood is very friable and the outlines of the piece of film cannot be distinguished clearly from the zone of migration. We therefore recommend the use of leukocyte film from human or cats' blood.

Although it is generally accepted that the hemopoietins act selectively on erythropoiesis, we considered that the changes observed in leukocyte cultures might be used as an index of hemopoietic activity. Before our own, many attempts have been made to utilize the changes in the leukocytes in order to determine the hemopoietic activity of liver preparations, which are also regarded as erythropoietic [6, 7, 9, 13]. It is difficult, in terms of the unitary theory of hemopoiesis, to admit that leuko- and thrombocytopoiesis are unaffected by influences causing changes in erythropoiesis.

Our investigations have shown that material active in relation to leukocytes in blood cultures possessed a definite stimulating action on erythropoiesis both in vitro and in vivo. Active gastric juice led to an increase in the number of dividing cells in marrow cultures, mainly cells of the red series, and speeded the conversion of some cell forms into others, accelerating in particular the fragmentation and extrusion of the nuclei of the erythronormoblasts [3]. In animals (dogs, rabbits), activation of erythropoiesis was observed under the influence of gastric juice. The number of dividing cells in the marrow, and often the total number of cells of the red series, was increased, their maturation was accelerated, and the peripheral blood indices were raised [4].

On the other hand, Linman and co-workers [10, 11] have recently shown that hemopoietins also possess a leukopoietic action, and we have verified this by our own experiments.

In parallel investigations of the hemopoietic activity of material in blood cultures using the migration indices, the incorporation of radioactive iron into the erythrocytes of intact rabbits, and by Marinone's method [12] (increase in the number of mitoses in the erythroblasts of hypophysectomized rats), we found that the results were in agreement.

The simplicity of the method described enabled us to make an experimental study of the hemopoietins in various conditions not amenable to other methods of investigation, and the complete agreement between our results and those described in the literature, obtained by more direct methods, convinced us that our method is suitable for such investigations.

According to our findings [1], this method may also be used for comparing the efficacy of liver preparations. This requires the simultaneous testing of several dilutions of the preparations, viz. 1:1000, 1:5000, and 1:10,000 (dilution is carried out when the cultures are set up). The most active preparation is characterized by the highest indices in a low concentration.

The suggested method has also been used successfully for the study of the toxic and antitoxic properties of serum from burns [5].

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