

A PHYSIOLOGICAL STUDY OF ERYTHROBLASTS IN THE DUCK

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MARKED changes in color indices, cell volume indices, and relative cellular hemoglobin indices have been observed by Rostorfer and Rigdon¹ in the blood of ducks during the course of severe malarial infections and during the period of recovery. A difference in the amounts of functional hemoglobin calculated from the oxygen capacity of the blood in comparison to the amounts of hemoglobin found by photo-electric colorimetry has been reported by Rigdon and Rostorfer² to occur in duck blood during malarial infection. Blood from malarial infected ducks has been shown by Rostorfer and McGee³ to have an abnormally low percentage of O₂ saturation at lowered pH levels compared to normal duck blood at the same pH levels. These observations have been correlated with the number of young red cells in the blood.



FIG. 1. This drawing represents the layers of red blood cells as observed following the centrifugation of blood from a duck recovering from malaria. A, layer of white cells; B, layer of erythroblasts; C, layer of erythrocytes and parasitized erythrocytes.

A chance observation made during the above physiological studies lead to a procedure for the isolation of young red cells from the blood of the duck. Two layers of red cells were observed following centrifugation of blood removed from ducks on the seventh day after being inoculated with *P. lophurae*. The double layer of red cells appeared in the centrifuged blood only of those ducks recovering from a severe *P. lophurae* infection. The upper layer of cells was lighter in color than the lower layer (fig. 1). Microscopic examination of the two layers showed that the upper layer was composed essentially of erythroblasts and the lower layer of adult red cells and parasitized red cells.

A quantitative separation of the layers proved to be possible and a study was made of the color index, cell volume index, relative cellular hemoglobin index,

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This work was supported by a grant from the John and Mary R. Markle Foundation. The anticoagulant used in these experiments was Lequaemin, supplied by Roche-Organon Inc., Nutley, N. J.

Research paper 836, Journal Series, University of Arkansas.

functional hemoglobin, oxygen dissociation curve, and oxygen utilization by erythroblasts in comparison to that of the adult red cells of the normal duck.

MATERIALS AND METHODS

Young white Pekin ducks 18 days old were inoculated with *P. lophurae* obtained from highly parasitized donor birds. The parasitemia was followed daily by counting the number of parasitized red cells per 500 red cells in blood smears stained with a combination of Wright's and Giemsa's stains. After the peak of parasitemia was reached and the number of parasitized cells was declining the young red cells were present in numbers between 250 and 400 per 500 red cells. At this stage of the infection one of the ducks was bled by heart puncture and the resulting heparinized sample was centrifuged at 1,500 rpm. for five minutes. If two layers appeared in the blood the remaining birds in the group were bled and the blood was pooled and centrifuged in 15 ml. centrifuge tubes. In order to obtain enough cells from the upper layer to make possible the analytical observations on hemoglobin, color index, oxygen capacity, and oxygen utilization studies a total of 100 ml. of blood was used for the separation of the upper from the lower layer. After the first centrifugation the upper layer of cells was carefully siphoned off along with approximately one half the plasma from each of the samples. These samples were pooled and recentrifuged. The upper half of the cells, following the second centrifugation, was siphoned from each tube and pooled. Centrifugation was repeated for the third time and the upper half of cells was siphoned off and suspended in plasma. These cells were centrifuged and the hematocrit reading was obtained. They were then resuspended and used for analytical purposes. Attempts were made to restore the hematocrit reading to the normal range; however, it was usually necessary to dilute the sample with plasma in order to have enough volume to carry out the analytical procedures.

Red cell counts were made and the color indices were calculated by dividing the amount of hemoglobin calculated from the oxygen capacity by the total cell count. The oxygen capacity was determined by equilibrating the sample with air at 38° C and analyzing by the use of the Van Slyke manometric apparatus. Colorimetric hemoglobin was determined by the method of Schultze and Elvehjem.⁴ Oxygen utilization per red cell was determined by equilibrating cells in a Warburg flask at 38° C in an atmosphere of pure oxygen. All cells were washed three times in physiological saline and finally suspended in saline for the determination of oxygen utilization.

Usually it was difficult to find ducks with a high percentage of young cells, since it is necessary for the birds to have a severe infection to develop a marked degree of anemia. A high percentage of the ducks died with this degree of infection.

RESULTS

Figure 1 illustrates the layers of red cells formed upon centrifugation of blood obtained from *P. lophurae* infected ducks sacrificed on the seventh day after inoculation. The upper layer represented approximately 30 per cent of the red cell volume.

In one experiment in which separation of the layers was attempted, the final concentration of the cells from the upper layer contained few parasitized cells and few adult erythrocytes. The percentages of cells in the different layers are shown in table 1. It can be seen from this table that the separation was nearly quantitative. It will be noted that the color index, relative cellular hemoglobin index, colorimetric hemoglobin and oxygen capacity were lower while the cell volume index was higher than corresponding values for normal duck blood and for the original malarial blood as well as for the sample obtained from the lower layer of centrifuged cells. The data for the lower layer are not normal with respect to the color index, which was somewhat higher than that for blood of the normal bird of similar age.

TABLE 1

	Malarial duck blood before separation or centrifugation	Sample from lower layer of cells	Sample from upper layer of cells
Total red cell count.....	693,000	704,000	605,000
Total parasitized red cells per 500 red cells...	335	355	73
Young red cells per 500 red cells.....	200	141	426
Parasitized young red cells per 500 red cells..		32	39
Color index.....		5×10^{-11}	1.6×10^{-11}
Cell volume index.....		1.4×10^{-11}	3×10^{-11}
Relative cellular hemoglobin index.....		2.8×10^{-1}	19×10^{-1}
Hematocrit.....	22%	22%	22%
Colorimetric hemoglobin log I ₀ /I.....		0.265	0.122
Oxygen capacity.....		4.76 vol. %	1.79 vol. %

In another experiment in which the separation of the two layers was complete, so that the final concentration of the cells from the upper layer contained no parasitized cells and only 5 to 7 adult red cells per 500 cells, the color index for the erythroblasts was only 1.9×10^{-11} . The values for normal duck blood in comparison with those found for the erythroblasts were as follows: color index of 3.7×10^{-11} , cell volume index of 1.4×10^{-11} , and relative cellular hemoglobin index 29×10^{-1} .

In one of the earlier attempts to separate the upper cell layer from the lower, an incomplete separation resulted. The final concentration of cells from the upper layer did contain an increased number of young red cells and a 50 per cent reduction in the number of parasitized cells when compared to the original blood. However, in this experiment enough sample was obtained to furnish a sufficient volume to enable the authors to determine the oxygen dissociation curves for the original blood and both the upper and lower layers. The percentages of oxygen saturation of the three samples equilibrated at an oxygen tension of 85 mm. Hg. and a CO₂ tension of 31 mm. Hg. were as follows: original blood from malarial infected ducks from which the other two samples were obtained was 70 per cent saturated; the sample containing the cells from the lower layer was 72.7 per cent saturated; while the sample containing the cells from the upper layer was only 62 per cent

saturated. Thus the dissociation curve for the sample containing the largest number of young red cells was lower than the curve for the original unseparated malarial blood. There was, however, a slight difference in CO_2 combining power between the three samples. The difference apparently was caused by the greater number of cells present in the original sample. Each sample was diluted with the same plasma. This difference amounted to less than one volume per cent of CO_2 and would not represent a sufficient change to cause a shift of the oxygen dissociation curve which represents a relatively large change of 8 per cent in O_2 saturation at 83 mm. of Hg. of oxygen tension.

A comparison of the utilization of oxygen by the normal adult red cell and the oxygen utilization by the erythroblasts was made by washing normal red cells in physiological saline three times and determining the oxygen consumption by placing approximately 760,000,000 cells suspended in 2.5 ml. of physiological saline in 15 ml. Warburg flasks. The gas space was filled with pure oxygen and the CO_2 evolved was absorbed in the usual manner. The flasks were shaken for 2 hours at 38°C and readings were taken every 15 minutes. The utilization of oxygen per red cell per hour was 2×10^{-8} cmm. This would correspond to a utilization of about 5 ml. of O_2 per 100 ml. of blood per hour or 5 vols. per cent per hour.

The utilization of oxygen by the normal red cells shaken in the Warburg flasks was checked against the utilization of oxygen by the normal red cells in an unshaken closed container in which normal blood equilibrated with air at 38°C was confined to an air-tight space and allowed to stand at 38°C for 25 minutes. The oxygen content of samples of this blood was compared with the oxygen capacity of a sample of the same blood freshly equilibrated with air at 38°C . The difference in the amount of oxygen of the two samples divided by the total cell count and the number of minutes of standing gave data from which the utilization of oxygen per cell per hour was calculated. This figure was 1.4×10^{-8} cm. of O_2 per cell per hour. This is slightly below the utilization of oxygen by the cells which were shaken in the Warburg flasks.

Blood obtained from young ducks on the seventh day after inoculation was centrifuged at 1,500 rpm for 5 minutes. Twenty seven per cent of the total cell volume was in the upper layer of cells. One hundred milliliters of this blood were separated as previously described and the final concentration yielded a sample of cells from the upper layer which was suspended in plasma obtained from a normal duck. These cells were uncontaminated with either parasitized cells or adult red cells. The cell count was 650,000, the color index 1.9×10^{-11} , and the oxygen capacity was 1.69 volumes per cent. From this sample aliquots of erythroblasts containing some 780,000,000 cells were removed and washed three times in physiological saline. They were suspended in 2.5 ml. of physiological saline and placed in each of several 15 ml. Warburg flasks and shaken at 38°C for 2 hours as described. The utilization of oxygen per young red cell was 8.6×10^{-8} cm. of oxygen per hour. This is over four times the rate of oxygen utilization by the normal red cell of the duck. If duck blood were composed entirely of erythroblasts and had a cell count of 2,500,000 per mm^3 , which is the normal red cell count of the normal young duck, the blood itself would use 20 volumes per cent of oxygen per hour.

DISCUSSION

Rostorfer and Rigdon¹ have observed an increase in cell volume index which was correlated with the presence of young red cells rather than with the degree of parasitemia. A decrease in color index and relative cellular hemoglobin index was observed also at the time of the greatest anemia which was correlated with the presence of erythroblasts rather than with the degree of parasitemia. These observations indicated that the young red cell was of larger volume and had less hemoglobin which was more loosely packed within the cell than in the case of the normal red cell. Measurements carried out by Rigdon and Rostorfer⁵ indicate that there was a relatively slight increase in mean diameter of the erythroblast over that of the normal red cell following the peak of parasitemia. There was a change from the nearly spherical shape of the very young erythroblast to the more elliptical shape of the normal red cell.

The erythroblast contained less hemoglobin, as indicated by the low color index. The hemoglobin content of these cells as measured by photo-electric colorimetry is not so low as calculations from the oxygen capacity would indicate. When the colorimetric readings (\log of I_0/I) were plotted against the oxygen capacity hemoglobin, the linear relationship was lower and to the right of the normal colorimetric and oxygen capacity hemoglobin ratio. This can be interpreted to indicate the presence of either iron or some iron-bearing compound within the cell which is not functional hemoglobin. The erythroblasts may have their normal iron component but an abnormally low hemoglobin content. However, the amount of nonfunctional pigment in the erythroblast is not present in sufficient amount to increase the color index to the normal range of the adult erythrocyte if this pigment should contain iron and develop into hemoglobin as the young cell matures. Several possibilities present themselves for consideration. First, the possibility that the colorimetric hemoglobin determination measures the presence of some compound which does not contain iron and which is in no way related to hemoglobin. The hemoglobin calculated from the oxygen capacity would then be the only approach to the hemoglobin content of bird blood. Thus the erythroblasts would be considered as having their full complement of hemoglobin without the prospect of gaining hemoglobin as they mature. They would be abnormal cells which would be only partly functional and their ultimate fate might be an early destruction. Such a destruction, however, has not been observed.

Second, the colorimetric hemoglobin determination might include both iron and iron bearing organic compounds which are related to or are precursor substances for hemoglobin. In this consideration the hemoglobin calculated from the oxygen capacity would represent only the functional hemoglobin present. Thus the erythroblast would be considered a true young red cell which had the potentialities of becoming an adult red cell with the normal color index, volume index, etc.

No iron determinations were made on the erythroblasts in this study. The iron content as measured by the Schutze and Elvehjem hemoglobin method would indicate the presence of some extra iron. However, as mentioned above, this extra

iron or pigment apparently is too small in amount to raise the color index to normal if this iron were formed into hemoglobin. It is inconceivable to us at this time that the erythroblast gains iron after its entry into the blood stream but such might prove to be the case. It has been said that iron in any form introduced into the blood stream cannot be transferred into red cells, since there can be no direct absorption.⁶

The erythroblasts are metabolically active. They consume four to five times the amount of oxygen used by the normal adult red cell. It is known that the amount of oxygen consumed varies with the age of the cell.⁶

Rostorfer and Rigdon¹ have observed in the duck that the young red cells disappear quickly following the peak of parasitemia. Their greatest number was present on the seventh day after the birds were inoculated and they were not to be found usually after the ninth day. The color index, volume index, relative cellular hemoglobin index were normal by the tenth or eleventh day following inoculation. The red cell count was normal also by the eleventh or twelfth day after inoculation. If all erythroblasts formed in the duck with malaria were of the type studied here they would have to gain a large amount of iron and hemoglobin in order to become normal adult red cells in the two or three days it requires the blood to reach the normal color index during the recovery from malaria. The possibility of these erythroblasts being only temporarily present in the blood stream and being destroyed early and then replaced by normal erythrocytes produced as such in the hematopoietic centers cannot be offset by the fact of the great oxygen utilization of the erythroblast.

Other types of severe anemias might be studied in the manner prescribed here. In fact, blood samples from a case of familial hemolytic jaundice were centrifuged and two layers of red cells were observed. Slides made from these layers were observed to contain distinctly different types of red cells.

SUMMARY

1. A method for the separation of erythroblasts in quantity from normal red cells in the blood of ducks infected with malaria has been described.
2. The erythroblasts in comparison to the normal adult red cells of the duck have a larger cell volume, less hemoglobin, and show a greater discrepancy between the colorimetric hemoglobin and hemoglobin calculated from the oxygen capacity.
3. The erythroblasts in the duck use four to five times the amount of oxygen that is consumed by the adult red cells in the ducks.
4. The function and possible fates of the erythroblasts which occur in the duck during the anemia caused by malaria are discussed.

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