Observations on the Nature of the Erythropoietic Serum Factor

By Allan J. Erslev and Paul H. Lavietes

The production of red blood cells has recently been shown to be influenced by a humoral factor found in blood plasma. The existence of such a factor was postulated in 1906 by Carnot and Delflandre. However, convincing experimental evidence for this was not obtained before Reis mann studied parabiotic rats in 1950 and Erslev bioassayed large amounts of plasma in 1953.

The present study was made to give some information as to the biochemical and biological nature of the humoral factor (erythropoietic factor) found in the plasma of bled rabbits. The effect of nitrogen mustard on the ability to produce this factor has also been investigated.

Materials and Methods

Adult, male, white New Zealand rabbits weighing 2.8 to 3.2 Kg. were used throughout as donors and recipients. They were fed red rose rabbit pellets and water and kept in an air-conditioned animal house.

In the first group of experiments, bioassays were made on serum, heparinized plasma and serum protein fractions obtained from normal and anemic donor rabbits. The anemic donors were bled from the heart repeatedly for periods of 24 hours to eight weeks and their hemoglobin concentration was at all times kept below 7 Gm. per cent. Serum was partially fractionated by salting-out with half saturated ammonium sulfate. It was diluted with an equal volume of water, and dialyzed in cellophane tubing against an equal volume of saturated ammonium sulfate plus a large volume of half saturated ammonium sulfate, with constant stirring. The precipitate was separated from the supernatant fluid by centrifugation, and both fractions were dialyzed in closed cellophane tubing against 0.85 per cent saline until free of sulfate. They were then both brought back to the volume of the original serum with 0.85 per cent saline. Paper electrophoretic studies revealed that the precipitate contained almost all the gamma globulin, while the supernatant fluid contained the remainder of the serum proteins.

In the second group of experiments, a standardized rapid bleeding technic was employed in order to allow evaluation of the effect of prior administration of nitrogen mustard. Fifty ml. of blood was removed and replaced by an equal volume of 0.85 per cent saline. Four hours later, 40 ml. more blood was taken and again replaced with saline. Forty-eight hours after the first bleeding, the animals were lightly anesthetized with 60 to 75 mg. of Nembutal and exsanguinated. Most animals yielded 90 to 120 ml. of whole blood. The serum was pooled for assay.

This serum served as a control for serum obtained from rabbits treated identically except that they were given nitrogen mustard by rapid intravenous injection 4 hours before the initial bleeding. The dose was either 5 or 10 mg. (1.7 to 3.3 mg. per Kg.) dissolved in 2 ml. of saline.

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Table 1.

<table>
<thead>
<tr>
<th>Range of reticulocyte count</th>
<th>Number of duplicate determinations</th>
<th>Standard error of mean of two observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%-3%</td>
<td>50</td>
<td>0.4%</td>
</tr>
<tr>
<td>3%-5%</td>
<td>50</td>
<td>0.5%</td>
</tr>
<tr>
<td>5%-7%</td>
<td>50</td>
<td>0.6%</td>
</tr>
<tr>
<td>7%-9%</td>
<td>20</td>
<td>0.9%</td>
</tr>
<tr>
<td>9%-11%</td>
<td>15</td>
<td>1.4%</td>
</tr>
</tbody>
</table>

In all experiments, normal healthy rabbits with a hemoglobin concentration averaging 13.7 Gm. per cent (ranging from 12.5 Gm. per cent to 15.0 Gm. per cent) and a reticulocyte count averaging 2.6 per cent (ranging from 1.4 per cent to 4.1 per cent) were selected as recipients.

For each assay, 200 ml. of serum or plasma, or fraction derived from a like amount of serum, was used. Fifty ml. was injected into the marginal ear veins of the recipient rabbit once a day for four days.

Hemoglobin determinations and reticulocyte counts were made on blood from the ears of the recipient rabbits on the day before the first injection (day -1), on the day of the first injection (day 0), on the day of the fourth injection (day 3) and on each of three following days (day 4, day 5 and day 6).

Hemoglobin concentrations were determined with a Coleman Junior Spectrophotometer on oxyhemoglobin.

Reticulocyte counts were made on dry cover glass smears prestained with brilliant cresyl blue and counterstained with Wright's stain. Duplicate counts were made by having two independent observers count 1000 red cells each.

The observational error of the reticulocyte counts was estimated from the standard error of the mean of the results from the two observers (4):

$$\text{S.E. of mean: } \frac{\text{Sum of Differences}}{N \times 2} / \sqrt{2}$$

(N is the number of duplicate determinations). The standard error of the mean was found to vary proportionally with the reticulocyte count (table 1).

In 100 duplicate determinations on 50 normal rabbits the mean reticulocyte count ±1 standard deviation was found to be 2.0 per cent ±0.7 per cent. The standard deviation was calculated from the formula:

$$\text{S.D.: } \sqrt{\frac{\text{Sum of squares of deviations from mean}}{N - 1}}$$

The statistical significance of a difference between mean reticulocyte counts was calculated by determining it from the formula:

$$t = \frac{\text{Difference between means}}{\text{Standard deviation}} \sqrt{\frac{N_1 N_2}{N_1 + N_2}}$$

($N_1$ and $N_2$ are the number of counts made in each of the two groups). The probability of $t$ was found from Fisher's table (4).

Results

I.

(a) Once a day for four days 34 recipient rabbits received each 50 ml. of serum or plasma from donors kept anemic for varying lengths of time (24 hours-8 weeks), but all with a hemoglobin concentration of less than 7 Gm. per cent.

Ten recipients received the same volume of normal serum or plasma from donors with a normal hemoglobin concentration.
The mean reticulocyte counts and their standard deviations on days -1, 0, 3, 4, 5 and 6 are plotted in figure 1. The serum or plasma from anemic donors induced a moderate but definite reticulocytosis while the normal serum or plasma had no significant effect on the reticulocyte count. The difference between the two curves on days 3, 4, 5 and 6 are statistically highly significant with P values of far less than 0.01.

(b) Of the 34 rabbits which received serum or plasma from anemic donors,
9 received heparinized plasma and 25 received serum. No significant difference between the reticulocyte response in these two groups was found.

(c) Four recipient rabbits each received 200 ml. of 0.85 per cent saline containing the gamma globulin from 200 ml. of serum from anemic donors.

Seven recipient rabbits each received 200 ml. of 0.85 per cent saline containing the serum proteins other than gamma globulin from 200 ml. of the serum.

Figure 2 gives the mean reticulocyte counts and their standard deviations in each group on days –1, 0, 3, 4, 5 and 6. From figure 1 and figure 2, it can be seen that there is no significant difference between the reticulocytosis induced by serum from anemic donors and the reticulocytosis induced by saline containing the albumin, alpha globulins and beta globulins from this serum. It can be seen from figure 2 that gamma globulin fails to induce a reticulocytosis.

(d) Serum kept at 4 C. or at –20 C. for 1–2 months appeared as active as freshly drawn serum.

II.

Five recipient rabbits each received 200 ml. of serum from donors injected with 1.7 mg. per Kg. of nitrogen mustard four hours before being made anemic. The average hemoglobin concentration of the donors at death was 5.7 Gm. per cent.

Three recipients each received 200 ml. of serum from donors injected with 3.3 mg. per Kg. of nitrogen mustard prior to being made anemic. The average hemoglobin concentration of these donors at death was 5.9 Gm. per cent. The mean reticulocyte response of the recipients in each of these two groups (1.7 mg. per Kg. and 3.3 mg. per Kg.) was almost identical. Figure 3 shows the mean reticulocyte response and the standard deviation calculated from the reticulocyte counts of all eight recipients.
Fig. 4. Reticulocyte response of 7 normal rabbits to serum obtained from healthy donor rabbits 48 hours after removal of 90 ml. of blood. Mean ±1 S.D.

As a control 7 rabbits each received 200 ml. of serum from donors which were made anemic in exactly the same way and kept anemic for the same length of time as the nitrogen mustard treated animals but without receiving this chemical. The average hemoglobin concentration of these donors at death was 6.5 Gm. per cent. Figure 4 shows the mean reticulocyte response and the standard deviation.

On days 3, 4, 5 and 6 the reticulocyte counts of these two groups (fig. 3 and fig. 4) did not differ in a statistically significant manner.

**Discussion**

The old hypothesis that blood plasma from anemic individuals contains a factor which stimulates red cell production has always appeared attractive but difficult to prove. Numerous investigators have attempted to demonstrate this factor (see reviews by Grant and Root (5) and by Hirsjärvi (6)), but the small amounts of serum or plasma used for bioassay and the lack of critical evaluation of the error inherent in counting red blood cells and reticulocytes have made the results conflicting and unconvincing. Conclusions based on these results, that the factor is a lipid or a serum globulin (7), that it has no relation to vitamin B 12 or to folic acid (8), or that it is formed in vitro by exposing blood to low barometric pressure (6, 9), have been equally unconvincing.

However, recent experiments with rabbits and monkeys (3, 10) have resulted in substantial experimental evidence for the existence in serum and plasma of bled animals of a factor capable of inducing reticulocytosis in normal animals. In rabbits (3) this reticulocytosis has been shown through simultaneous red blood cell count, hematocrit determinations and bone marrow studies, to reflect an increased erythropoietic activity of the bone marrow.

At present, reliable information in regard to the nature of this humoral erythropoietic factor can be obtained only through the bioassay of quantities of
serum and plasma which approximate the total blood volume of the recipient animal. The administration of these large volumes of fluid makes the bioassay technic very crude. However, valid conclusions can be drawn if a sufficient number of bioassays and careful control studies are performed.

The studies reported here have given some information as to the nature of the erythropoietic factor. It has been shown to be non-dialysable and to remain in solution during the transformation of fibrinogen to fibrin and during salt precipitation of gamma globulin. It is furthermore stable at room temperature, at 4 C. and at −20 C. These findings seem to indicate that the erythropoietic factor is attached to or behaves like a serum albumin, alpha globulin or beta globulin.

When nitrogen mustard is administered to laboratory animals degenerative cellular changes occur in the bone marrow within 8 hours (11). These toxic changes have usually been held solely responsible for the depressed red blood cell production. However, it seems possible that nitrogen mustard in addition might interfere with the production of the erythropoietic serum factor. In support of this hypothesis, Jacobson and coworkers have reported (12) that bone marrow regeneration, after nitrogen mustard administration, is enhanced if splenic tissue is protected by ligation of the splenic pedicle during and shortly after nitrogen mustard is administered. This experiment seems to indicate that nitrogen mustard impairs the production in the spleen of a substance capable of stimulating hematopoietic tissue. However, evidence for the existence of such a factor is inferential. The authors suggest as alternative explanations for their observations that the intact spleen may neutralize toxins or supply cells to migrate to the bone marrow.

In order to study the possibility that nitrogen mustard impairs the production of erythropoietic factor, 1.7 mg. per Kg. or 3.3 mg. per Kg. was administered to donor rabbits. Graef et al. (11) have reported that LD50 doses, 2–3 mg. per Kg., will depress reticulocyte formation and cause cytotoxic changes in the normoblastic tissue. The administered nitrogen mustard was given ample time to exert its toxic effect before the donors were made anemic by bleeding. “Anemic” serum was obtained 48 hours later. In view of the possible effect of length of time in which the animals were kept anemic and the degree of anemia on the production of the erythropoietic factor, a control group of donor animals was bled in exactly the same way but without first receiving nitrogen mustard. The average hemoglobin concentration of the control group at death was slightly higher than in the nitrogen mustard treated group.

Bioassay of serum from the two groups revealed no significant difference in the reticulocyte response. The conclusions from this experiment seem to be (a) that nitrogen mustard has no effect on the production of erythropoietic factor, (b) that the factor probably is not produced in lymphatic, hematopoietic or other nitrogen mustard sensitive tissues and (c) that it is not related to the bone marrow stimulating substance which Jacobson has suggested might be produced in the spleen.

**Summary**

Serum and plasma from anemic rabbits were found to contain a factor capable of inducing a reticulocytosis when injected into normal animals. This eryth-
Erythropoietic factor seems to be attached to, or to behave like, a serum albumin,
alpha globulin or beta globulin. The production of the erythropoietic factor was found to be normal in rabbits in which the lymphatic and the hematopoietic tissues had been damaged through the administration of nitrogen mustard.

**Summario in Interlingua**

Esseva constatate que le sero e le plasma ab conilios anemic contine un factor que pote inducer reticulocytosis quando illo es injeite in animales normal. Il pare existir un relation—un attachamento directe o un similaritate de action)—inter iste factor erythropoietic e un albumina seral o un globulina alpha o beta.

Le production del factor erythropoietic se revelava como normal in conilios in que le texitos lymphatic e hematopoietic habeva essite ledite per le administration de HN₂ hydrochlorido.

**REFERENCES**

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