Studies on the Erythropoiesis Inhibiting Factor in the Plasma of Animals with Transfusion Polycythemia

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IT HAS BEEN proved by many authors that polycythemia induced by blood transfusion reduces the peripheral reticulocyte count and inhibits erythropoiesis.1-10

In previous investigations on erythropoiesis we have observed that polycythemia causes the appearance in the plasma of an active substance, which is antagonistic to erythropoietin. The plasma obtained from polycythemic animals was injected into normal ones, and the influence of these injections on the recipients has been described. We have named the active substance "erythropoiesis inhibitor."11

In the present paper further results of experiments on the factor inhibiting erythropoiesis are reported.

METHODS

1. Preparation of concentrated filtrates of polycythemic and normal plasma: Polycythemia was induced in sheep weighing 45-55 kg. by triple transfusion of the red cells of normal sheep in the form of an 80 per cent suspension in saline. The suspension, 600-800 ml., was given intravenously on each of three consecutive days. As anticoagulant heparin, Leo (made in Denmark) was used. The level of polycythemia obtained in sheep reached 170-190 per cent of the standard taken prior to the transfusion (pre-transfusion hematocrit was 34.2 ± 3.5 per cent; after transfusions, it attained 61.2 ± 5.0 per cent). Blood was taken from polycythemic sheep 24 hours after the last transfusion. The centrifuged plasma will be referred to as "polycythemic plasma". This was adjusted to pH 5.5 with 1 N HCl, then deproteinized by keeping it in a boiling water bath for 10 minutes. The filtrate was concentrated at 40 C. One ml. of the concentrated filtrate corresponded to 10 ml. of the original plasma volume. The filtrate was adjusted to pH 7.2 with 1 N NaOH. Concentrated filtrates of normal plasma were prepared in the same manner.

2. Preparation of concentrated filtrates of polycythemic plasma from nephrectomized sheep: Sheep were nephrectomized bilaterally and polycythemia was induced by triple transfusion of red cells as stated above. The general condition of the animals was good four days after nephrectomy. No major changes were observed in the respiratory or cardiac rhythm in the sheep. They were lively, responsive, and had a good appetite. The blood urea level before nephrectomy was 60 mg. per cent; after 24 hours—100 mg. per cent; after 48 hours—150 mg. per cent; after 72 hours—220 mg. per cent; and after 96 hours—280 mg. per cent. (In our previous investigation the tested sheep were in good general condition eight days after nephrectomy, despite the high rise of urea in their blood). The first transfusion was given immediately after nephrectomy. Blood was taken and centrifuged 24 hours after the last red cell transfusion. The plasma filtrate was prepared in the way described above.

3. Preparation of concentrated plasma filtrate from sheep with blockade of reticuloendothelial system: Blockade of reticuloendothelial system was induced in sheep by three intravenous injections at 24 hourly intervals of 1 per cent of trypan blue in amounts of 0.1 Gm./Kg. of body weight. Blood was taken and centrifuged 24 hours after the last injection. The plasma was prepared as described above.

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4. **Blood and bone marrow examinations:** The concentrated filtrates of polycythemic and normal plasma were injected subcutaneously in 31 rabbits, in amounts of 0.8 ml. daily for 10 days. The rabbits were normal adult mongrels of both sexes in weight from 2200-2800 Gm. The effect of the injections on the blood and bone marrow was determined by RBC and reticulocyte counts and measurement of hemoglobin and hematocrit, as well as by bone marrow biopsies before and after the experiment. RBC counts were made in Burcker counting chambers. In every case two blood samples were drawn into Thoma pipettes, diluted and counted. A one per cent water solution of Nile blue was used for the supravital staining of reticulocytes. The reticulocytes were counted on each smear until a total of 2000 erythrocytes had been recorded. Hemoglobin was estimated as basic hematin using a Zeiss photocolorimeter. The Wintrobe hematocrit was centrifuged for one hour (at 3000 rpm.). The marrow samples were obtained by biopsy from the tuber ischiaticus or os ileum. The freshly drawn marrow smears were made in the same manner as blood smears and stained with May-Grunwald-Giemsa stain.

5. **Incorporation of Fe\(^{59}\):** The concentrated filtrates of polycythemic and normal plasma, of polycythemic plasma obtained from nephrectomized sheep and of plasma from sheep with blockade of the reticuloendothelial system were injected subcutaneously in rats in the amount of one ml. daily for three days. The rats were of the Wistar strain of both sexes ranging in weight from 160-200 Gm. Six hours after the last injection of the plasma filtrates, each rat received one µg of Fe\(^{59}\). Standards were prepared at the time the isotope was administered by mixing known amounts of blood and Fe\(^{59}\). Sixteen hours after injection, blood was withdrawn by cardiac puncture and the incorporation of Fe\(^{59}\) into red cells was determined. The total blood volume in rats was assumed as six per cent of the body weight.

6. **In vitro tests for hemolysis:** A test for hemolysis in rabbits was performed in vitro with polycythemic and normal plasma. Polycythemic or normal sheep plasma filtrate, 0.5 ml., in 1:1, 1:100, 1:1000, and 1:10000 dilutions was mixed with fresh guinea pig plasma or rabbit plasma in 1:10 dilution in an amount of 0.3 ml. and with a three per cent suspension of rabbit red cells in an amount of 0.5 ml. The test tubes were kept at 37 C. In the control test rabbit, plasma or saline replaced the polycythemic plasma filtrate.

**RESULTS**

1. **Influence of the concentrated filtrate of polycythemic sheep plasma on erythropoiesis in rabbits and rats:** Figure 1 presents the hematologic data on rabbits receiving the filtrate of polycythemic plasma (15 rabbits) and those that were injected with filtrate of normal plasma (16 rabbits). The filtrate of polycythemic plasma diminished the red cell count on the average by 700,000 per cu.mm. Hematocrit values decreased on the average by 4 Gm. hematocrit points and hemoglobin values on the average by one Gm. The reticulocyte count rose from 0.11 per cent to 0.18 per cent. The differences between hematologic data before and after injections of concentrated filtrate of polycythemic plasma were statistically significant (for erythrocytes p < 0.0001, for hematocrit, hemoglobin and reticulocytes p < 0.001). Injection of the concentrated filtrate of normal plasma produced no significant change in the hematologic data.

Bone marrow examinations of rabbits receiving the filtrate of polycythemic plasma showed that the number of erythroblastic cells in marrow smears decreased from 32.6 per cent before, to 22.3 per cent after the injections. Since the erythroblasts were counted in proportion to the remaining cells (i.e., chiefly cells of the leukopoietic system) the number of leukocytes in the circulating blood was examined before and after the experiment. No signif-
Fig. 1.—The hemoglobin value, hematocrit, red cell count and reticulocyte count in rabbits injected with the concentrated filtrate of the polycythemic and normal plasma.

Significant differences were found, the mean being 7,800 WBC before and 8,000 WBC after injections of the filtrate. No changes in leukocyte precursors in the rabbit bone marrow and in the morphology of circulating leukocytes were found either. On the basis of these findings it is assumed that the reduction of the percentage of erythroblastic cells in marrow smears was the consequence of an absolute reduction of their number in the bone marrow.

Differential marrow counts of erythrocyte precursors in rabbits injected with the filtrate of polycythemic plasma were also performed. They were based on 500 erythroblasts in each rabbit and are given in figure 2. The character of the maturation curves remained essentially unchanged. The differences indicated in figure 2 are statistically significant in the case of erythroblasts in division (p = 0.0474), but in other cases "p" was larger than 0.05.

In order to prove that decrease of the number of erythrocytes was indeed due to the inhibition of erythropoiesis and not to hemolysis after injection of the filtrate of polycythemic sheep plasma, rabbit erythrocytes were tested in vitro for hemolysis by the filtrates from polycythemic plasma. Fresh guinea pig or rabbit plasma was used as complement. The tests were made by commonly used methods and confirmed that after one, two and four hours no hemolysis of rabbit blood occurred, regardless of the dilutions used.
Twenty-five rats were used for the study of the effects of the polycythemic plasma filtrate on Fe\textsuperscript{59} incorporation. The uptake of Fe\textsuperscript{59} in rats receiving the filtrate of polycythemic plasma reached 21.2 per cent of the injected amount while the control animals receiving the filtrate of normal plasma incorporated 42.3 per cent (table 1). The experiments with Fe\textsuperscript{59} proved that the triple injections of filtrate of polycythemic plasma (in amounts equivalent to 30 ml. of polycythemic plasma administered to each rat) caused inhibition of Fe\textsuperscript{59} uptake by erythrocytes.
Table 1.—Fe¹⁹ Uptake After Injections of the Concentrated Filtrate of Plasma Received from Experimental Sheep

<table>
<thead>
<tr>
<th>Injections</th>
<th>Number of rats</th>
<th>Per cent of Fe¹⁹ incorporation into rats' red cells</th>
<th>Standard deviation of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated filtrate of normal sheep plasma</td>
<td>16</td>
<td>42.3 ±7.0</td>
<td></td>
</tr>
<tr>
<td>Concentrated filtrate of plasma from sheep with transfusion polycythemia</td>
<td>25</td>
<td>21.2 ±4.1</td>
<td></td>
</tr>
<tr>
<td>Concentrated filtrate of plasma from sheep with blockade of the reticuloendothelial system</td>
<td>25</td>
<td>25.3 ±7.8</td>
<td></td>
</tr>
<tr>
<td>Concentrated filtrate of plasma from sheep with transfusion polycythemia induced after earlier bilateral nephrectomy</td>
<td>20</td>
<td>28.7 ±5.3</td>
<td></td>
</tr>
</tbody>
</table>

If the results obtained from the control animals are adopted as 100 per cent, it may be assumed that after the injections of polycythemiac plasma filtrate the inhibition of erythropoiesis reached 50 per cent.

2. Influence of concentrated filtrates of the polycythemic plasma obtained from bilaterally nephrectomized sheep on erythropoiesis in rats: The plasma filtrate obtained from polycythemic bilaterally nephrectomized sheep was injected to rats in the amount of one ml. daily for three days. The erythropoietic activity in rats was determined using Fe¹⁹. The results are presented in table 1. The "p" value is lower than 0.001 for the difference between the percentage of Fe¹⁹ uptake after injections of concentrated filtrates of normal plasma and the percentage of Fe¹⁹ uptake after injections of concentrated filtrates of plasma received from sheep with transfusion polycythemia induced after earlier bilateral nephrectomy.

Comparison of the results obtained in both groups of rats showed that inhibition of erythropoiesis was produced by the injections of polycythemiac plasma filtrate obtained from sheep in which transfusion polycythemia was induced following bilateral nephrectomy.

3. Influence of the concentrated filtrate from sheep with blockade of the reticuloendothelial system on erythropoiesis in rats: The plasma filtrate obtained from sheep with blockade of the reticuloendothelial system was injected subcutaneously into rats in an amount of one ml. daily for three days. The activity of erythropoiesis in rats was determined with Fe¹⁹. The results are presented in table 1, and indicate marked inhibition by the plasma from the sheep with blockade. The "p" value is lower than 0.001 for the difference between the percentage of Fe¹⁹ uptake after injections of concentrated filtrates of normal plasma and the percentage of Fe¹⁹ uptake after injections of concentrated filtrates of plasma received from sheep with blockade of the reticuloendothelial system. The degree of inhibition of erythropoiesis in rats injected with filtrates from sheep after blockade of the reticuloendothelial system is similar to that obtained with the filtrate of polycythemiac plasma.
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DISCUSSION

The present experiments confirm previously published data on the “erythropoiesis inhibitor.” These data indicate that the action of the inhibitor consists in (a) considerable reduction of the percentage of Fe$^{59}$ uptake into red cells; (b) reduction of the erythroblastic cells in the bone marrow; (c) reduction of the percentage of erythroblast mitosis in the bone marrow; (d) reduction of red cell count, hematocrit and hemoglobin in the circulating blood.

It is difficult to explain the percentage rise of reticulocytes in the circulating blood with simultaneous decrease of the red cell count, hemoglobin and hematocrit level, and reduction of erythroblast percentage in the bone marrow. In our previous paper$^{11}$ the statistically significant decrease of reticulocyte counts in rabbits after three intraperitoneal injections of the polycythemic plasma was established. In the experiments presented above we injected the polycythemic plasma filtrate subcutaneously over a 10 day period.

The inhibiting action of the erythropoiesis inhibitor on erythropoiesis was confirmed by a 50 per cent reduction of the normal incorporation of Fe$^{59}$ into red blood cells of rats injected subcutaneously with the concentrated filtrate of polycythemic plasma.

It has been demonstrated that the polycythemic plasma obtained from bilaterally nephrectomized sheep also contains the erythropoiesis inhibitor. Blockade of the reticuloendothelial system, using trypan blue in sheep, induced production of a substance similar or identical with the erythropoiesis inhibitor, obtained after transfusion polycythemia.

In present experiments we have not taken into account the amount of “cold iron” because in earlier unpublished experiments we established that the level of cold iron in the filtrate of polycythemic plasma reducing erythropoiesis was not higher than that in the filtrate of normal plasma. This indicates that the presence of cold iron in the filtrate of polycythemic plasma has no influence on the results obtained with radioactive iron.

SUMMARY

Further investigations of the action of polycythemic plasma filtrate were made using Fe$^{59}$ and with detailed examination of the blood and bone marrow. These studies confirmed the appearance of an active thermostable plasma factor (erythropoiesis inhibitor) which depressed erythropoiesis in normal rabbits or rats. The plasma obtained from bilaterally nephrectomized sheep subjected to transfusion polycythemia also contained the erythropoiesis inhibitor. Blockade of the reticuloendothelial system using trypan blue, in sheep, induced production of the active substance similar or identical with the erythropoiesis inhibitor produced after transfusion polycythemia.

SUMMARIO IN INTERLINGUA

Esseva effectuate investigationes additional con respecto al action de filtrato de plasma polycythemic. Le investigationes utilisava Fe$^{59}$ e includeva un detaliate examine del sanguine e del medulla ossee. Esseva confirmate le ap-
partition de un active factor plasmatic de character thermostabile (inhibitor de erythropoiese) que deprimeva le erythropoiese in conilios o rattos normal. Le plasma obtenite ab bilateralmente nephrectomisate oves subjicite a poly
cythemia de transfusion etiam contineva le inhibitor de erythropoiese. Le bloque
age del systema reticuloendothelial per medio de blau trypan induceva in
oves le production de un substantia active simile a (o identic con) le inhibitor
del erythropoiese producite post polycythemia de transfusion.

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