STUDIES ON RED CELL APLASIA, I. DEMONSTRATION OF A PLASMA INHIBITOR TO HEME SYNTHESIS AND AN ANTIBODY TO ERYTHROBLAST NUCLEI

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A cell culture system1, 2 in which human bone marrow responds to the in vitro addition of erythropoietin with increased heme synthesis provides a new means of studying disorders of red cell production, and has already been used to study the erythropoietic response of marrow from patients with polycytemia vera.3 This method should also be useful in the investigation of those diseases of underproduction of red cells where enough marrow can be obtained to perform the study. Such a disease is pure red cell aplasia in which the marrow has a normal cellularity with only a deficient number of red cell precursors. Several investigators4-7 have suggested that this disease is due to an autoimmune state, but little evidence of a circulating inhibitor to erythropoiesis has so far been presented. Our studies of a patient with red cell aplasia show that his marrow not only responds to added erythropoietin in vitro with an increased rate of heme synthesis, but also develops an enhanced control level of heme synthesis when separated from high concentrations of his plasma. In addition, the patient’s plasma inhibits heme synthesis of normal marrow in vitro, and contains an antibody to erythroblast nuclei. These findings, taken with the patient’s recovery to a normal state after treatment with 6-mercaptopurine, an immunosuppressive agent, all lend support to the idea that this disease may be due to a circulating antibody.

Materials and Methods.—The patient (M. E.) was a 45-year-old white male from San Jose, Costa Rica, who was well until September 1962, when progressive anemia was first noted. He took no medications up to this time, and the only contacts with toxic agents were an exposure to insecticides for 7 years and to dinitrophenol for 4 months. Laboratory tests revealed a normochromic, normocytic anemia with a complete absence of reticulocytes in the peripheral blood. Platelet and white cell counts were normal. Bone marrow aspiration showed an almost complete absence of normoblasts. The patient was treated with 100 μg of vitamin B12 intramuscularly (IM) each day for 1 month; then folic acid, 15 mg/day, for 2 weeks; then depo-testosterone, 100 mg IM every 15 days, and prednisone, 40 mg/day, for 2 months; then vitamin B12, 50 mg IM, and prednisone, 10 mg, for 7 months. During this latter period, in June 1964, he had a splenectomy which revealed a normal spleen. The patient then received oxymetholone, 100 mg daily, for 6 months; then cobalt IM daily for 1 month; then riboflavin, 20 mg by mouth, for a similar period. Neither the above medications nor the splenectomy helped his anemia so that during this period he received several units of blood every 4 to 5 weeks.

In January 1966 he was referred to the University of Chicago Hospitals. Physical examination revealed a short, lean man who appeared chronically ill. A darkened skin and mild hepatomegaly were the only positive physical findings. The blood picture showed a severe anemia without any reticulocytes, a white cell count of 6,850 cells/mm³ with a normal differential smear, and a platelet count of 510,100 cells/mm³. Smears and sections8 revealed a slightly hypercellular bone marrow with normal white cell and platelet precursors but a marked decrease in red cell precursors. Stainable iron was present in excessive amounts and many small lymphocytes were present. Chest X ray showed no evidence of thymoma. Plasma iron was 286 μg % and total iron binding capacity was 486 μg %. Plasma protein immunoelectrophoresis was normal. Latex fixation, sheep cell agglutination, and anti-DNA titers were negative. Chromosome analysis of the bone marrow
showed a normal male karyotype. Plasma and urine erythropoietin titers were measured by Dr. Clifford Gurney with the polycythemic mouse assay and were extremely high. The Fe\(^{59}\) plasma disappearance time was markedly prolonged, and iron kinetics showed virtually no marrow uptake of Fe\(^{59}\) into new red cells.

The bone marrow cell cultures were prepared as previously described,\(^1\) by passing the marrow through a pipette several times to disperse the cells and then filtering the suspension through a sterile stainless steel mesh to remove loose bone particles. The dispersed cells were washed twice with 5 ml of NCTC-109 or Hanks' balanced salt solution (Microbiological Associates) and were then added to 35 \(\times\) 10 mm tissue culture dishes (Falcon Plastics) in an aliquot of NCTC-109 or Hanks' solution with 50 units of penicillin and 170 units of heparin/ml. Fe\(^{59}\) was added and radioactive heme extracted and counted as in the previous reports.\(^1\) Sheep plasma erythropoietin (Step III, lot K147 192A) was obtained from the U.S. Public Health Service Study Section on Hematology.

For the cytoimmunofluorescence study, the patient's IgG globulins were extracted from his plasma by the cold ethanol fractionation technique at a temperature of \(-5\)°C.\(^9\) The method of Wood, Thompson, and Goldstein\(^1\) was used to conjugate the IgG globulin with fluorescein isothiocyanate (Baltimore Biological Laboratory) and to purify the conjugated globulin with DEAE cellulose. The second eluate obtained from the DEAE cellulose column with 0.03 M sodium phosphate buffer was used for the present study.

Bone marrow cells were washed three times with 5\% albumin in isotonic phosphate-saline buffer, pH 7.4, and smears were made and air-dried. The slides were fixed in absolute methanol for 5 min, washed in the buffered saline solution for 15 min, flooded with fluorescein-conjugated IgG for 30 min, washed in three changes of buffered saline for a total of 30 min, and mounted in buffered glycerol. The preparations were examined with a Zeiss GFL microscope equipped with a dark field condensor and a General Electric AH6 mercury arc lamp. After the fluorescent fields were recorded, the slides were washed in the buffered saline solution for 2 hr and then dried and stained with Wright-Giemsa.

**Results.**—When the marrow from this patient was incubated in 20 per cent normal human plasma with erythropoietin, the rate of heme synthesis increased threefold above the rate of the untreated cells by 72 hours (Fig. 1). Moreover, the rate of heme synthesis of the untreated controls, which has repeatedly declined with other bone marrows,\(^1\),\(^2\) increased twofold by 66–72 hours as compared to 0–6 hours. This increase in the controls could be due to residual endogenous erythropoietin, but we have never observed it in marrow from other patients with hemolytic anemias incubated under the same conditions. When the same marrow was incubated in 20 per cent of the patient's own plasma, the controls without erythropoietin increased to a greater rate by 66–72 hours than with incubation in normal plasma, and addition of erythropoietin had no effect. This was probably because the erythropoietic titer of the patient's plasma was already so high that the added

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**Fig. 1.—Effect of erythropoietin on marrow of patient with red cell aplasia.** Cultures were started with 738 nucleated cells/mm\(^3\) in a total volume of 0.8 ml. Solid line indicates cultures which had 20\% normal plasma, 20\% newborn calf plasma, and 60\% NCTC-109. Broken line shows cultures which had 20\% patient's plasma instead of normal plasma. The controls (\(\bullet\) and \(\triangle\)) had no added erythropoietin; stimulated (\(\bigcirc\) and \(\triangle\)) had 0.3 units/ml. Each point is the mean of duplicates and indicates the middle of a 6-hr incubation with 0.8 \(\mu\)c Fe\(^{59}\)/ml.
amount was not significant. Nevertheless, this experiment indicated that under the proper conditions this patient’s marrow could increase its rate of heme synthesis by 6.5 times over its starting rate when placed in vitro.

From these data it appeared that the marrow was either freed from some in vivo inhibitory factor or that a new essential factor was supplied in the in vitro medium. The patient’s marrow was therefore incubated in 89 per cent of his own plasma and in 33 per cent of his own plasma diluted with Hanks’ salt solution (Table 1). The rate of heme synthesis did not increase over 78 hours when the medium consisted of 89 per cent autologous plasma, but increased sixfold when the patient’s plasma was diluted with a simple salt solution. Since this salt solution contained nothing that could conceivably have created this effect, this suggested that the patient’s plasma must contain some factor which inhibited this increase of heme synthesis. A normal plasma control was not a part of this experiment, but dilution with calf serum did not maintain an inhibition, but rather stimulated the rate of heme synthesis about 23-fold over the initial 6-hour rate. This effect of calf serum in augmenting the stimulation of heme synthesis by erythropoietin has been noted.\(^1\)

In another experiment with the patient’s marrow a normal plasma control was run: Increased concentrations of normal plasma did not inhibit the rate of heme synthesis, but a high concentration of the patient’s plasma was associated with a

**Table 1**

<table>
<thead>
<tr>
<th>Marrow medium</th>
<th>Time (hr)</th>
<th>μMoles heme/6 hr</th>
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<tbody>
<tr>
<td>33% Patient’s plasma</td>
<td>0–6</td>
<td>2.28</td>
</tr>
<tr>
<td>67% Hanks’ salt solution</td>
<td>72–78</td>
<td>13.2</td>
</tr>
<tr>
<td>89% Patient’s plasma</td>
<td>0–6</td>
<td>4.22</td>
</tr>
<tr>
<td>11% Hanks’ salt solution</td>
<td>72–78</td>
<td>3.45</td>
</tr>
<tr>
<td>33% Patient’s plasma</td>
<td>0–6</td>
<td>2.52</td>
</tr>
<tr>
<td>56% Newborn calf serum</td>
<td>72–78</td>
<td>58.9</td>
</tr>
<tr>
<td>11% Hanks’ salt solution</td>
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Cultures were started with 1,100 nucleated cells/mm\(^3\) in a total volume of 0.9 ml. No exogenous erythropoietin was added to any of the cultures. Fe\(^{69}\) was added at 0.7 μc/ml at 0–6 hr in some cultures and after 72 hr of incubation for another 6 hr in replicate cultures. Each value is the mean of duplicates.

In Fig. 2.—Effect of dilution of patient’s plasma and normal plasma on heme synthesis by (A) patient’s marrow and (B) normal marrow. (A) Cultures were started with 658 nucleated cells/mm\(^3\) in a total volume of 0.9 ml medium which consisted of NCTC-109 and either the patient’s plasma (---) or normal plasma (——). Fe\(^{69}\) was added after 69 hr of prior incubation for a period of 6 hr before terminating the experiment. (B) Cultures were started with 445 nucleated cells/mm\(^3\) in a total volume of 1.0 ml medium which consisted of NCTC-109 and either the patient’s plasma (---) or normal plasma (——). Fe\(^{69}\) was added after 66 hr of incubation for 8 hr. In both experiments all dishes had 0.3 units/ml of erythropoietin. The points are the mean of duplicates and represent an incubation with 0.43 μc of Fe\(^{69}\).
50 per cent decline (Fig. 2A). When the patient’s plasma was titrated against the marrow of another person in vitro, it showed a 50 per cent inhibition of the rate of heme synthesis at a concentration of 90 per cent (Fig. 2B), while a normal plasma control again showed no inhibition. Repeated experiments like this one have shown that the rate of heme synthesis by normal cells with increased concentrations of normal plasma fluctuated from one experiment to another; the patient’s plasma, however, inhibited the rate of heme synthesis by at least 50 per cent of the control value at the highest plasma concentrations.

When the rate of heme synthesis of the patient’s marrow was tested with high concentrations of his plasma at 0–6 hours and at 72–78 hours, the inhibitory effect was only observed at the later time (Fig. 3). This also occurred when his plasma was added to normal marrow. The increased concentrations of the patient’s plasma therefore did not inhibit heme synthesis already in progress, but prevented the increase in the rate of heme synthesis observed 72–78 hours later. Thus the plasma inhibitor is apparently not a simple chemical inhibitor of heme synthesis but probably acts to prevent the later effects of erythropoietin on increased heme synthesis.

Because of the possibility that this inhibitor was an antibody, purified IgG globulins were prepared from the patient’s plasma (Fig. 4) and conjugated with fluorescein isothiocyanate. This conjugated globulin caused an apple-green speckled fluorescence of many round nuclei in normal marrow cells (Fig. 5A). When these cells were counterstained with Wright-Giemsa, they were easily identified as erythroblasts (Fig. 5B). The leukocyte precursors showed only a green cytoplasmic fluorescence, perhaps due to antibodies formed through frequent blood transfusions, while fresh cryostat sections of liver and lymph nodes, and lymph node imprints, showed no green nuclear fluorescence. Additional controls consisted of an unstained bone marrow smear, one pretreated with the patient’s unlabeled IgG and then stained with the fluoresceinated IgG, and a bone marrow smear stained with a similarly prepared, fluorescein-labeled IgG from normal plasma. None of these showed the green nuclear fluorescence, demonstrating the specificity of the reaction between the patient’s conjugated IgG and erythroblast nuclei.
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Fig. 5.—(A) Application of patient’s fluorescein-labeled IgG globulin to normal marrow cells demonstrating green fluorescence of two round nuclei. (B) This was followed by application of Wright-Giemsa stain demonstrating that both cells were erythroblasts.

Figure 6A shows the patient’s initial course in the University of Chicago Hospitals and demonstrates a complete absence of reticulocytes in a man whose hematocrit regularly declined and was brought back to normal only by repeated blood transfusions. The first four months of his hospitalization were devoted to the aforementioned studies and the collection of plasma as a working material. Because he had not been given large doses of steroids after splenectomy, 80 mg of prednisone was begun daily as a first form of treatment. This medication appeared to decrease his transfusion requirement but did not produce reticulocytes, and was therefore tapered to maintenance doses.

Since the preceding evidence suggested that the circulating plasma inhibitor to heme synthesis might be an antibody and since 6-mercaptopurine (6-MP) has been used with moderate success in other diseases associated with antinuclear antibodies,14 the patient was next started on this medication at 2.2 mg/kg, after giving his informed consent. Within one week the first positive reticulocyte count appeared at 1.1 per cent (Fig. 6B). The reticulocytes remained stable for another week but then began to fall. The 6-MP dose was then raised in an attempt to further increase the reticulocytes. These, however, soon fell to zero, followed by a decline in the white cell and platelet counts, and the development of a toxic hepatitis. It was evident that 6-MP had produced a bone marrow depression, and its use was discontinued. Subsequently the platelet and white cell counts returned to normal, followed by a massive recovery of the hemopoietic tissue with a 9.1 per cent reticulocytosis and disappearance of the toxic hepatitis. A subsequent bone marrow examination showed a normoblastic hyperplasia. The patient went on to attain a normal hematocrit, which, despite frequent phlebotomies as treatment for his hemosiderosis, he continued to maintain. No further therapy was administered and he left the hospital feeling quite well. Two months later he still maintained a normal hematocrit and antinuclear antibodies could no longer be demonstrated.

Discussion.—These experiments demonstrate a plasma factor which, in high concentration, inhibits the in vitro effect of erythropoietin on the marrow cells. This inhibition could be due to an action on the hormone itself, but it is difficult to explain how dilution of the patient’s plasma would allow erythropoiesis
to increase in those experiments where no hormone was added, since both hormone and inhibitor in the plasma were diluted equally with respect to the cells. Moreover, erythropoietin was present in the patient's plasma and urine in large, unneutralized amounts. The inhibitor therefore probably does not act on erythropoietin, but on the cells themselves, either by preventing the intermediate action of the hormone so that few newly differentiated erythroblasts are formed, or by destroying these newly formed cells as they are made. Further work is now necessary to characterize this inhibitor and to demonstrate its mechanism of action.

We have also shown that the patient's plasma contains an anti-erythroblast nuclear antibody, but this does not mean that this antibody is the principal inhibitor to heme synthesis. Until it can be shown that a pure preparation of this substance inhibits heme synthesis in cell cultures, we cannot be sure of this point. Nevertheless, others have shown that the earliest recognizable erythroid cell, the proerythroblast, already has specific antigen incorporated into it, and that
specific antibodies can be produced against lymphocytes which reduce their number and function.\textsuperscript{16} It is therefore entirely possible that the antibody to the erythroblasts demonstrated here is responsible for the \textit{in vitro} findings and thereby the patient's disease.

The idea that an antibody to marrow cells might cause this disease is not new, since a high association of red cell aplasia with thymoma has been reported, and in one fourth of these cases thymectomy has resulted in disappearance of the disease.\textsuperscript{4} Because the thymoma cells did not invade the bone marrow locally, they were thought to act via a circulating plasma factor, although this was never demonstrated. In 1949 Smith reported a case of pure red cell aplasia in a newborn associated with the formation of an anti-A agglutinin made by the mother in an incompatible pregnancy, and he postulated that this might be the cause of the red cell aplasia.\textsuperscript{7} Eisemann and Dameshek\textsuperscript{6} and Gasser\textsuperscript{4} have noted the occurrence of red cell aplasia with autoimmune hemolytic anemia and have postulated an extension of the antibody action on the red cells to the red cell precursors. The former authors reported the disappearance of both after splenectomy.\textsuperscript{8} Entwistle \textit{et al.}\textsuperscript{18} found a circulating plasma inhibitor to the erythropoiesis of rabbits in a man with red cell aplasia and a carcinoma of the bronchus. This inhibition disappeared with resection of the carcinoma but was never fully characterized. Jepson and Lowenstein\textsuperscript{15} demonstrated a plasma inhibitor to mouse erythropoiesis in two patients with red cell aplasia and postulated that this might be an anti-erythropoietin, but their data can equally well be interpreted as the production of an antimarrow factor. Holborow \textit{et al.}\textsuperscript{18} and Barnes\textsuperscript{19} have described antinuclear factors in several patients with thymoma and refractory anemia but the functional specificity of these factors has not been determined. Professor Barnes has recently indicated\textsuperscript{20} that he may be able to demonstrate an \textit{in vitro} immunoglobulin inhibitor of erythropoiesis in patients with refractory anemia. Finally, several authors have injected animal marrow from one species into another to produce an antimarrow serum.\textsuperscript{21–22} When they then injected this antimarrow serum into the marrow donor species, a marrow aplasia occurred, suggesting that the production of an antimarrow factor could produce red cell aplasia. These studies were never extended to the complete characterization of an antibody or to human disease. Therefore, despite a consistent idea and much work, an antimarrow antibody has never been proved to be the cause of red cell aplasia.

Our studies describe the application of a marrow cell culture system to a particular disease, and show that this system may be very useful in deciding whether red cell aplasia is due to an anti-marrow cell antibody. This would not mean that all red cell aplasias must be due to the same cause. Certainly anti-erythropoietin antibodies have been demonstrated on mice\textsuperscript{24} and could arise spontaneously in humans. Also metabolic deficiencies specific to red cell precursors might occur which could cause this disease, so that red cell aplasia may be due to different causes in different cases just as there are different causes of hemolytic anemia. Nor should every plasma that inhibits heme synthesis \textit{in vitro} necessarily be implicated as the cause of the patient's disease, since tissue culture systems are notoriously sensitive to extraneous, nonspecific factors. The positive finding of increased heme synthesis by the marrow with time of incubation \textit{in vitro} may be a more reliable index of the patient's disease when coupled with the demonstration of a plasma inhibitor to this increase of heme synthesis.
Nevertheless, our data suggest that this disease could be due to a specific circulating antibody to the marrow erythroblasts which acts either to prevent their development or to destroy them as fast as they arise. This would explain why stimulators of erythropoiesis such as cobalt or androgens might help to overcome the disease since they could produce more erythropoietin and thus more erythropoietic cells than there is inhibitor, and why anti-antibody measures like splenectomy, steroids, and 6-MP may work since they could reduce the quantity of the inhibitor. It also explains all the in vitro data reported here. We should be able to ascertain the validity of this hypothesis in the near future.

Summary.—When the marrow from a patient with red cell aplasia was treated with erythropoietin in vitro, there was a marked increase in the rate of heme synthesis over the starting rate. This increase could be prevented by high concentrations of the patient’s plasma, which inhibited the cellular response to the hormone. An antibody to erythroblast nuclei was demonstrated in the patient’s plasma, and treatment with 6-mercaptopurine, which removed this antibody, was associated with disappearance of the patient’s disease. These experiments suggest that this disease may be due to a circulating antibody to the marrow erythroblasts.

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† Operated by the University of Chicago for the U.S. Atomic Energy Commission.
6 Gasser, C., Sangre (Barcelona), 26, 6 (1955).