Studies on Red Cell Aplasia. II. Report of a Second Patient with an Antibody to Erythroblast Nuclei and a Remission After Immunosuppressive Therapy

By SANFORD B. KRANTZ AND VINCENT KAO

RED CELL APLASIA is an anemia characterized by a marked reduction in the number of marrow erythroblasts.1,2 Although cases have been reported in which the blood leukocyte or thrombocyte count was also reduced, the marrow usually has a normal cellularity, normal granulopoiesis and normal megakaryocytopoiesis.1,2 While red cell aplasia is a somewhat rare disease, only 150 cases having been reported, it has attracted an extraordinary amount of interest and several extensive reviews of this subject have been written in the last decade.1,3 The disease is often associated with thymomas and there is a prevailing idea that it may be due to an antibody which interferes with the process of erythropoiesis.1-5 We have previously reported a case of red cell aplasia in which a plasma inhibitor to heme synthesis and an antibody to erythroblast nuclei were present.13 When this patient’s marrow was separated from the plasma and was incubated in vitro a marked enhancement of the rate of heme synthesis occurred. These results suggested that the patient’s disease might be due to a circulating antibody directed against the marrow erythroblasts. The patient was treated with an immunosuppressive drug, 6-mercaptopurine, and shortly thereafter a reticulocytosis occurred and a normal hematocrit was achieved which persisted one year later. Since spontaneous remissions occur in this disease it is important to confirm these results. This paper describes a second case of red cell aplasia with many similar findings and a response to immunosuppressive therapy.
CASE REPORT

L.G., a 54 year old white female, was in good health until January, 1961 when the onset of anemia was noted. She was receiving no medications at the time but had been exposed to insecticides. The family history was noncontributory and physical examination revealed no significant abnormalities. Laboratory tests showed a normochromic, macrocytic anemia, 0.1 per cent reticulocytes, and a normal platelet count. The white cell count was 4,200 cells per cu. mm. with a normal differential count. The bone marrow was hypercellular but had virtually no polychromatophilic or orthochromatic erythroblasts. Some proerythroblasts which appeared megaloblastic were present. In all other respects the marrow was normal. Direct and indirect Coombs tests were negative. The Schilling test for vitamin B\textsubscript{12} absorption was normal. Roentgenograms of the chest were normal.

The patient was given vitamin B\textsubscript{12} 100 micrograms per week, folic acid, 15 milligrams per day, intramuscularly (IM), and Valentine's liver extract, 90 ml. per day by mouth. When no reticulocyte response was noted by 19 days, prednisone was begun at 40 mg. per day and the vitamins were discontinued. Eight days later the reticulocytes increased to 2.8 per cent and the patient began to maintain a normal hematocrit. The prednisone was reduced slowly over the next 4 months to 10 mg. per day. At this dosage the hematocrit began to decline and it was soon evident that 30-40 mg. of prednisone per day was necessary to maintain a normal hematocrit. Bone marrow examination during prednisone administration showed a hypercellular marrow with an abundance of mature erythroblasts.

The prednisone was discontinued in November, 1963 after the occurrence of multiple vertebral compression fractures. At this time two lupus erythematosus cell preparations were negative. A red cell survival time using \textsuperscript{51}Cr was 23½ days. On electrophoresis the serum proteins were albumin 3.3 Gm. per cent and globulin 2.6 Gm. per cent. The \gamma-globulin was 1.0 Gm. per cent. The bone marrow showed little evidence of erythropoietic differentiation, only an occasional proerythroblast being noted. Fluoxymesterone, 30 mg. per day by mouth, was administered for one month. This was followed by testosterone enanthate, 600 mg. IM twice per week, for two months. No erythropoietic response was noted and androgen therapy was therefore discontinued. During this period plasma and urinary erythropoietin levels were extremely high as measured by the polycythemic mouse assay.

A splenectomy was performed in February, 1964. Microscopic examination of the spleen showed lymphoid hyperplasia and hemosiderosis. Serum electrophoresis demonstrated an albumin of 3.2 Gm. per cent and globulins of 2.7 Gm. per cent. The \gamma-globulin was 1.2 Gm. per cent and appeared to be largely within a narrow peak suggesting that a paraprotein might be present. The serum was anticomplementary. Bence-Jones proteins were not present in the urine. Bone marrow examination revealed a normocellular marrow with virtually no erythroblasts and only a slight increase in plasma cells.

No erythropoietic response to splenectomy occurred by June, 1964 and the administration of prednisone was resumed. The patient was maintained for 2½ years on 60 mgm. of prednisone every other day with estradiol valerate, 20 mgm. IM per month, but hyperglycosuria, salt retention, and heart failure occurred during this period. In Feb., 1967 it was apparent that this regimen was no longer sufficient to maintain a normal hematocrit. Laboratory tests showed an apparent serum complement level of less than 5 units per ml. Serum anticomplementary activity was again noted. Further study of the plasma revealed the presence of pyroproteins which precipitated after the plasma was heated to 60 C. for 30 minutes, and did not redissolve upon cooling. A 2 per cent pyrocrit was present, while the cryoglobulin test was negative. Serum electrophoresis demonstrated an albumin of 2.9 Gm. per cent and globulins of 2.7 Gm. per cent. The \gamma-globulin was 0.4 Gm. per cent and a paraprotein was clearly present which was 1.1 Gm. per cent. This paraprotein, demonstrated by immunoelectrophoresis to be a \gamma-G-globulin, was probably present at the time of the first serum electrophoresis in 1963. Roentgenograms of the bones demonstrated no osteolytic lesions. Bone marrow examination again revealed a normocellular marrow with virtually no mature erythroblasts and no myeloma cells. In vitro studies of the patient's erythropoietic tissue were begun at this time.
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MATERIALS AND METHODS

The bone marrow cell cultures were prepared as previously described. The dispersed cells were transferred to 35 X 10 mm. tissue culture dishes in an aliquot of NCTC-109 and then heparinized human plasma or sterile-filtered, precolostrum, newborn calf serum were added. Heparin and penicillin were added at a final concentration of 100 units per ml. and 30 units per ml., respectively. ^Fe in the form of ferric chloride was attached to transferrin by incubation with human plasma at 37 C. overnight. 0.1 ml. of this radioiron solution was added to the cultures at intervals and radioactive heme was later extracted and counted as in the previous reports. The Geiger counter* had a background of 1.5 to 2.0 counts per minute. Sheep plasma erythropoietin concentrate, 3.3 units per mg. of protein (Step III, lots K147 192A and K147 187), was obtained from the U.S. Public Health Service Study Section on Hematology. Human urinary erythropoietin concentrate, 65 units per mg. of protein, was obtained from Dr. Frederick Stohlman, Jr.

The G-globulins were extracted from the plasma and purified by the standard techniques of ammonium sulfate precipitation and DEAE cellulose column chromatography. One half of the purified G-globulin was prepared for incubation with marrow cells in vitro. It was dialyzed against Hanks' balanced salt solution and then applied to a Sephadex G-25 column and eluted with the Hanks' solution. The concentration of the G-globulin was measured by its refractive index. It was then adjusted with Hanks' solution to the original concentration of the G-globulins in the plasma. All glassware and dialysis tubing used in preparation of this fraction were cleaned as described by Paul. The other half of the G-globulin was conjugated with fluorescein isothiocyanate and then purified on a DEAE cellulose column as described by Wood, Thompson, and Goldstein.

The conjugated G-globulins obtained from this second DEAE cellulose column by elution with .05 M sodium phosphate buffer were used for the present study. The procedure for applying the fluorescein-conjugated globulins to slides of normal marrow cells and then counterstaining with Wright-Giemsa stain was described in the previous report.

Marrow cell nuclei were prepared by washing the cells twice with 5 per cent albumin in isotonic sodium phosphate-buffered saline, pH 7.4. The cell pellet obtained by centrifugation was then homogenized in a Potter-Elvehjem homogenizer with .01 M sodium phosphate-buffered saline, pH 7.4. This homogenate was centrifuged at 10,000 X g. for 5 minutes and the nuclei were washed twice with the same buffer. After the final pellet was resuspended, only cell nuclei could be seen with the light microscope.

Immunoelectrophoresis was performed on ionagar plates in .025 M veronal buffer using polyvalent immunoglobulin antiserum as well as specific rabbit or goat antisera for detecting human G-globulin, and kappa and lambda light polypeptide chains.

RESULTS

When the patient's marrow was incubated in vitro with erythropoietin the rate of Fe incorporation into heme increased fourfold over the initial rate by 50 hours (Fig. 1). The rate of Fe incorporation into heme also increased in marrow cells that were incubated without erythropoietin, though not to the same degree. These results are similar to those observed in a previous case of red cell aplasia, but are in contrast to the results observed with normal human marrows, where the rate of heme synthesis declined continuously when erythropoietin was not present. They are also in contrast to the results with bone marrows from patients with primary refractory anemia where the

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†Pharmacia Fine Chemicals, Piscataway, New Jersey.
‡Baltimore Biological Laboratory, Baltimore, Maryland.
§Hyland, Los Angeles, California.
¶Calbiochem, Los Angeles, California.
‖NCI Immunoglobulin Reference Center, Springfield, Virginia.
Fig. 1.—Effect of erythropoietin on marrow of patient with red cell aplasia. Cultures were started with 1,900 nucleated cells/mm³ in a total volume of 0.85 ml. Cultures had 24 per cent normal human plasma, 24 per cent newborn calf serum and 52 per cent NCTC-109. The controls (●) had no added erythropoietin; stimulated cultures (○) had 0.6 units/ml. of urinary erythropoietin. Each point indicates the middle of a 6 hr. incubation with 0.62 μc. ⁵⁹Fe.

Fig. 2.—Effect of erythropoietin on marrow of patient with sideroachrestic refractory anemia. The patient was an 82 year old Negro female who had been severely anemic for 10 years with an average reticulocyte count of 2 per cent. Her serum erythropoietin level was elevated, and her bone marrow showed an erythroid hyperplasia with ringed sideroblasts. Cultures were started with 2,300 nucleated cells/mm³ in a total volume of 0.85 ml. Cultures had 24 per cent of the patient’s plasma, 24 per cent newborn calf serum and 52 per cent NCTC-109. The controls (▲) had no added erythropoietin; stimulated cultures (△) had 0.3 units/ml. of urinary erythropoietin. Each point indicates the middle of a 4 hr. incubation with 0.30 μc. ⁵⁹Fe.
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Fig. 3.—Erythropoietin dose-response curve for marrow of patient with red cell aplasia. Cultures were started with 3,000 nucleated cells/mm³ in a total volume of 0.77 ml. Cultures had 20 per cent normal plasma, 20 per cent newborn calf serum and 60 per cent NCTC-109. Urinary erythropoietin was used and 1.17 µc ⁵⁹Fe was added after 70 hours of incubation, for 7 hours before terminating the cultures.

Table 1.—Effect of Patient’s Plasma on Heme Synthesis by Patient’s Marrow

<table>
<thead>
<tr>
<th>Marrow Cultures (No.)</th>
<th>Normal Plasma (%)</th>
<th>Patient’s Plasma (%)</th>
<th>Heme ⁵⁹Fe × Medium Fe (c.p.m.) % of Normal</th>
<th>Inhibition of Heme Synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>88</td>
<td>0</td>
<td>100 ± 8.57*</td>
<td>20 (0.20&gt;p&gt;0.10)</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>88</td>
<td>80 ± 1.45</td>
<td></td>
</tr>
</tbody>
</table>

* Standard error of the mean.

This table presents the means of four separate experiments performed in a similar manner. Cultures were begun with 305-1,150 nucleated cells/mm³ in 0.8 ml plasma and 0.1 ml NCTC-109. All cultures had 0.3 units/ml of sheep erythropoietin. After 44-70 hours of incubation, 0.48-1.24 µc ⁵⁹Fe was added for a period of 4-7 hours before terminating the cultures. The heme c.p.m. was multiplied by the total amount of iron present in the medium of each culture to correct for variations of unlabelled iron in the plasmas. The mean of this product for the cultures with normal plasma in each of the 4 experiments was expressed as 100 in order to compare the separate experiments.

The rate of heme synthesis declined continuously even when erythropoietin was added (Fig. 2). The effect of increasing concentrations of erythropoietin on this patient’s marrow was studied (Fig. 3) and this experiment demonstrated a threefold increase in the rate of heme synthesis over nontreated controls. The slight decline in heme synthesis that was observed at the very highest concentration of erythropoietin is believed to be due to a contaminant in the erythropoietin preparation.¹⁴

The fact that the rate of heme synthesis increased after incubation of the marrow in vitro suggested that the suppression of erythropoiesis in vivo might
Fig. 4.—Immunoelectrophoresis of patient’s plasma and γG-fraction. 1, 3, 4. Patient’s plasma. 2. Patient’s γG-globulins purified by ammonium sulfate precipitation and DEAE cellulose column chromatography.

be due to a plasma inhibitor as reported in the previous case. When high concentrations of the patient’s plasma were incubated with her marrow, however, heme synthesis was inhibited to only a slight, statistically insignificant, degree (Table 1).

The patient’s γG-globulins were purified to test for an antibody to erythroblast nuclei. Figure 4 shows that the patient’s paraprotein was a γG-globulin that was present with the normal γG-globulins in the purified preparation. The paraprotein had lambda-light polypeptide chains but no kappa-chains (Fig. 4). The fluorescein-conjugated γG-globulins were applied to slides of normal marrow cells and produced a speckled fluorescence of many round nuclei (Fig. 5A). Counterstaining of these cells with Wright-Giemsa stain identified them as erythroblasts (Fig. 5B). The granulocytes showed only cytoplasmic fluorescence (Fig. 5C). When the conjugated γG-globulins were applied to slides of marrow cell nuclei large numbers of round nuclei fluoresced (Fig. 5D) while polymorphonuclear leukocyte nuclei did not fluoresce. Granulocyte cytoplasmic fluorescence was not present in these nuclear preparations except in a very few cells, where a faint border of fluorescence appeared around nonfluorescing nuclei. When the marrow cells were pretreated with the patient’s unconjugated γG-globulins before applying the conjugated fraction, or when the marrow cells were treated with fluorescein-labeled γG-globulins from normal plasma, no nuclear fluorescence was observed.

Immunoelectrophoresis of the patient’s fluorescein-conjugated γG-globulins
showed that this protein was predominantly concentrated in a single band (Fig. 6). No precipitin reaction with antibody to \( \gamma \)-M- or \( \gamma \)-A-globulins was evident and no additional precipitin band appeared with rabbit polyvalent immunoglobulin antiserum. Ouchterlony immunodiffusion demonstrated that this fraction had both kappa- and lambda-light polypeptide chains (Fig. 6) and therefore contained some \( \gamma \)-G-globulins which were not paraproteins.

A preparation of the patient’s \( \gamma \)-G-globulins which had not been conjugated with fluorescein isothiocyanate was added to normal marrow cells in vitro. This markedly inhibited heme synthesis compared to similar preparations obtained from normal plasma and from the patient’s plasma after a remission had been induced (Table 2). In this experiment the final concentration of the patient’s purified \( \gamma \)-G-globulins was higher than the concentration of globulins extracted from normal plasma because of the presence of the paraprotein.
Fig. 6.—Immunoelectrophoresis and Ouchterlony immunodiffusion of patient’s fluorescein-conjugated \(\gamma\)G-globulins. 1. Patient’s plasma. 2. Patient’s fluorescein-labelled \(\gamma\)G-globulins. 3. Goat antiserum to human \(\kappa\) chains. 4. Rabbit antiserum to human \(\lambda\) chains.

The concentrations of the non-paraprotein \(\gamma\)G-globulins from the patient’s plasma and normal plasma were similar, however.

The patient’s hematologic course during treatment with immunosuppressive drugs is shown in Figure 7 which demonstrates a complete lack of reticulocytes and repeated need for regular blood transfusions. Throughout this study the patient was given 10 mg. of prednisone and 0.5 Gm. of tolbutamide per day. Azathioprine was administered initially but a pronounced decline in the platelet count and white cell count was evident after three weeks, and the drug had to be discontinued. The number of reticulocytes did not increase at this time or during the subsequent 18 weeks. After this period the administration of cyclophosphamide was begun. No increase in the number of reticulocytes was noted during the eighty-two days of treatment with cyclophosphamide. The white cell count decreased to a persistent low level of 2,000 cells per cu. mm. and the platelet count consistently remained at 100,000 cells per cu. mm. The bone marrow was very hypoplastic and the administra-
Fig. 7.—Course of patient during treatment with azathioprine and cyclophosphamide.
Table 2.—Effect of Patient’s Purified γG-Globulins on Heme Synthesis by Normal Marrows

<table>
<thead>
<tr>
<th>Marrow Cultures (No.)</th>
<th>Source of γG-Globulins</th>
<th>Concentration of γG-Globulins (mg./ml.)</th>
<th>Heme 59Fe (% of Normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Normal Plasma</td>
<td>0.85</td>
<td>100 ± 4.9*</td>
</tr>
<tr>
<td>5</td>
<td>Patient’s Plasma (Pre-treatment)</td>
<td>2.60</td>
<td>26.7 ± 5.9</td>
</tr>
<tr>
<td>7</td>
<td>Patient’s Plasma (After Remission)</td>
<td>3.20</td>
<td>111.4 ± 6.7</td>
</tr>
</tbody>
</table>

* Standard error of the mean.

This table presents the means of two similar experiments. Cultures were begun with 1,350 and 1,800 nucleated cells/mm³ in a total volume of 1.1 ml. with 0.6 ml. normal plasma and 0.25 ml. NCTC 109. The remainder of the medium consisted of the purified γG-globulins in Hank’s balanced salt solution. The concentration of the γG-globulins is the average value for both experiments. After 42 hours of incubation, 0.8 μc. of 59Fe was added for 27 hours before terminating the cultures. The mean heme c.p.m. for the cultures which had the normal purified γG-globulins was expressed as 100 in each experiment. The heme c.p.m. of the cultures with the patient’s purified γG-globulins was then expressed as a percentage of the normal value. No correction for unlabelled iron was made here since the added γG-globulins contained no significant concentration of iron.

Discussion

The observations in the present case of red cell aplasia are similar to those reported in a previous case. A marked enhancement of heme synthesis occurred when these marrows were incubated in vitro, and the marrow cells responded to the addition of erythropoietin with an even greater increase in heme synthesis. Although the data are presented in terms of heme synthesis, previous work has indicated that most of the heme radioactivity is derived from hemoglobin. Thus, separating the marrow of these patients with red cell aplasia from the in vivo environment appears to free the cells from an inhibitor and to promote a marked increase in hemoglobin synthesis.

As in the previous case of red cell aplasia, an antibody to erythroblast nuclei was found. In this case the nuclei were separated from most of the cytoplasm before applying the fluorescein-labeled antibody in order to demonstrate clearly that the antibody was not directed against the cytoplasmic membrane. This antibody appears to be a γG-globulin with a specific attrac-
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In our study, we detected an antibody for erythroblast nuclear antigen, since no fluorescence of white cell nuclei was observed. It was not found in normal plasma, nor in the plasma of the patient following treatment and remission of the disease. It is conceivable that this antibody could arise as a result of the destruction of erythroblasts in vivo and thus be a reflection of the disease, but not its cause. However, in the present case while we could not demonstrate an inhibitor to erythropoiesis in the patient's whole plasma, we did find that a fraction of the plasma, which contained the patient's purified yG-globulins, markedly inhibited heme synthesis by normal marrow cells. After treatment of the patient and the onset of a remission the inhibitor was no longer present. This suggests that an antibody directed against marrow erythroblasts may be interfering with red cell production in this disease. Other investigators have also indicated that they may be able to demonstrate an immunoglobulin inhibitor of erythropoiesis in patients with refractory anemia. The difficulty of demonstrating that the intact plasma contained an inhibitor to erythropoiesis in this case as compared to the previous case could be due to a lesser concentration of plasma inhibitor and/or to interference from other factors in the plasma (such as erythropoietin and anticomplementary activity). The total production of such an inhibitor may not always be reflected in the plasma, just as the plasma iron does not always indicate the true state of iron metabolism. In addition to a production-destruction equilibrium determining the plasma concentration there may be a plasma-cell equilibrium like that observed in hemolytic anemias where a direct or indirect Coombs test may reveal an anti-red cell antibody. Since marrow erythroblasts are greatly reduced in this disease the plasma remains the chief source for identification of the antibody, and because of the above factors plasma fractionation may have to be utilized to demonstrate the inhibitor.

The role of the patient's paraprotein is not entirely clear. Since the paraprotein had only one type of light chain, and since its concentration remained stable near 1 Gm. per cent for over four years, the patient appeared to have a “benign monoclonal gammopathy.” In addition the patient had no Bence-Jones proteinuria and no cytologic or roentgenographic evidence for multiple myeloma. One previous report of red cell aplasia with a benign I_gammopathy and one report of the coexistence of red cell aplasia with I_A multiple myeloma exist. The fact that the paraprotein gave no other evidence of interference with cellular functions over several years and that it persisted after the red cell aplasia had disappeared seems to indicate that it was not the principal causative factor in the red cell aplasia. The fluorescein-conjugated yG-globulins had both kappa- and lambda-light polypeptide chains, indicating the presence of yG-globulins which were not paraproteins. Further purification and characterization of the yG-globulins is necessary to clarify this point and to demonstrate the exact nature of the inhibitor, its relation to the fluorescein-conjugated antibody to erythroblast nuclei, and its mechanism of action. Nevertheless, from these data and the work previously described it appears that red cell aplasia may be due to a specific antibody to marrow erythroblasts, and that it may be successfully treated by immunosuppressive therapy.
SUMMARY

When the marrow from a patient with red cell aplasia was incubated in vitro a marked increase in heme synthesis occurred. Heme synthesis was further increased by the addition of erythropoietin. The patient's plasma contained both a γG-antibody to erythroblast nuclei and a γG-fraction that inhibited heme synthesis. After the patient was treated with cyclophosphamide, the antinuclear antibody and the inhibitor to heme synthesis disappeared and erythropoiesis was normal. These experiments confirm our previous study and indicate that red cell aplasia may be due to an antibody to marrow erythroblasts.

SUMMARIO IN INTERLINGUA

Quando le medulla ab un patiente con aplasia erythrocytica esseva incubate in vitro, il occurreva un marcate augmento in le synthese de hem. Iste synthese esseva augmentate additionalmente per le addition de erythropoietina. Le plasma del patiente contineva tanto un anticorpore γG a le nucleos erythroblastic como etiam un fraction γG que inhibiva le synthese de hem. Post que le patiente habeva essite tractate con cyclophosphamida, le anticorpore antinuclear e le inhibitor del synthese de hem dispareva, a le erythropoiese esseva normal. Iste experimentos confirma nostre previe studios e indica que aplasia de erythrocytos es possiblemente causate per un anticorpore anti erythroblastos medullari.

REFERENCES


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