Studies on Red Cell Aplasia

V. PRESENCE OF ERYTHROBLAST CYTOTOXICITY IN γG-GLOBULIN FRACTION OF PLASMA

SANFORD B. KRANTZ, W. H. MOORE, and S. DONALD ZAENTZ

From the Departments of Medicine, Vanderbilt University School of Medicine and Veterans Administration Hospital, Nashville, Tennessee 37203

Abstract The marrow cells of a patient with pure red cell aplasia markedly increased their rate of heme synthesis when they were freed from the host environment and were incubated in vitro. When the red cell aplasia was treated with cyclophosphamide and prednisone, marrow cell incorporation of 55Fe into heme in vitro increased several weeks before a reticulocytosis was apparent, and was the earliest effect noted. The plasma γG-globulins of this patient inhibited heme synthesis by normal marrow cells or the patient's own marrow cells obtained after remission of the disease.

Since the inhibition of heme synthesis could be the result of damage to erythroblasts, the patient's post-treatment marrow cells or normal marrow cells were labeled with 55Fe and were then incubated with the patient's pretreatment, treatment, and posttreatment γG-globulins as well as normal γG-globulins. At the end of incubation the supernatant and cells were separated and counted. Heme was extracted and also was counted. Treatment of the cells with the patient's pretreatment γG-globulins resulted in a release of 40% of the radioactive heme from the cells. This represented the loss of radioactive hemoglobin and was an index of erythroblast cytotoxicity. A progressive disappearance of the cytotoxic factor in the γG-globulins occurred in the 3 wk period preceding the onset of reticulocytes in the patient's blood. Posttreatment and normal γG-globulins did not produce this effect and increased injury of red cells and lymphocytes was not produced by the patient's pretreatment γG-globulins. These studies demonstrate a method for measuring erythroblast cytotoxicity and show that red cell aplasia is associated with γG-globulins that specifically damage erythroblasts. Whether interference with new erythroblast development also occurs and contributes to the inhibition of heme synthesis has not yet been ascertained.

Introduction

Pure red cell aplasia is an anemia characterized by an almost complete absence of marrow erythroblasts and blood reticulocytes (1, 2). Other hematopoietic functions such as granulopoiesis and megakaryocytopoiesis appear to be normal, and the marrow has a normal cellularity (1, 2). This disease is frequently associated with thymomas (1, 3-7) and has responded to thymectomy (1, 3, 4, 7), splenectomy (2, 8, 9), treatment with corticosteroids (2-4, 7), or with cytotoxic immunosuppressive drugs (10-15). Antinuclear antibodies (5, 10, 11, 14, 16, 17) and inhibitors of erythropoiesis (10, 11, 14, 16, 18, 19) have been reported in the plasma of some patients, and a few cases have had a coexistent Coombs positive hemolytic anemia (2, 8, 20). Because of these observations it has been suggested that pure red cell aplasia might be due to an antibody that prevented normal red cell production (1-3, 5, 6, 8, 10-12, 15-20).

We have studied this disease by using a marrow cell culture method in which human marrow responds to the addition of erythropoietin with a marked increase in heme synthesis (21, 22). These investigations demonstrated an inhibitor of heme synthesis and an antibody to erythroblast nuclei in the γG-globulin fraction of patients with pure red cell aplasia (10, 11, 15). After remission of the disease was produced by immunosuppressive drugs, both factors were no longer present (10, 11, 15). While these observations support the general idea that the disease may be due to an antibody to marrow erythroblasts they do not indicate the precise mechanism by which erythropoiesis is suppressed.
Inhibition of heme synthesis could be due to the prevention of new erythroblast development in vitro or could result from damage to previously formed erythroblasts. By withdrawing blood on a patient prior to treatment and then successfully treating the disease, we produced a situation in which the patient's pretreatment, treatment, and posttreatment plasma yG-globulin fractions could be tested on normal marrow, or on his own posttreatment marrow which had an erythroid hyperplasia. Our studies demonstrate that the patient's pretreatment yG-globulins inhibited heme synthesis by his own marrow cells in vitro. In addition, the patient's pretreatment yG-globulins, when incubated with his own marrow cells that had been prelabeled with "Fe, increased the release of labeled hemoglobin into the medium. When the patient was treated with cyclophosphamide and prednisone the erythroblast cytotoxicity of his yG-globulins progressively disappeared in the 3 wk period before the onset of a reticulocytosis. These experiments demonstrate that erythroblast injury was produced by the patient's yG-globulins prior to treatment and suggest that the reduced erythropoiesis of red cell aplasia might occur through production of this cytotoxic factor.

METHODS

Case report

The patient (E. M.), a 62 yr old white male, was well until August 1969 when he developed a sinusitis and was found to be anemic. A diagnosis of pure red cell aplasia was made after an examination of the bone marrow revealed virtual absence of erythroblasts with a normal cellularity, normal granulocytopenia, and normal megakaryocytopenia. The patient had a complete absence of reticulocytes in his blood, but had a normal white cell concentration and slightly elevated platelet concentration. There was no family history of this disorder and no extraordinary contact with toxic chemicals. Physical examination was normal except for a surgical aphakia and glaucoma. Roentgenograms of the chest including laminograms, did not reveal a thymoma. He was treated with 200 mg of pyridoxine and 30 mg of halotestin per day by mouth for 3 months. This was followed by testosterone enanthate 1,200 mg im/wk and prednisone 40 mg/day by mouth for another 3 months. At this point the testosterone was discontinued and the patient was treated with 50 mg of prednisone every other day for 4 months, followed by 100 mg every other day for another 4 months. Prolonged treatment with corticosteroids was associated with the onset of glycosuria and a peripheral neuropathy and the prednisone was reduced to 10 mg every other day.

No hematological response was noted with this treatment and in November 1970 the patient was referred to the Vanderbilt University Medical Research Center. The physical examination was unchanged except for a diffusely enlarged thyroid and slight hepatomegaly. A chest roentgenogram showed slight widening of the superior mediastinum which was believed to represent an enlarged thyroid gland after visualization by an "I" scan and aortogram. The blood pH was 7.4 with a white cell concentration of 6,500/µl with a normal differential count. The platelets were 456,000/µl, but the hematocrit was 21.6% and no reticulocytes were present. Bone marrow smears and particle sections again showed an almost complete absence of erythroblasts. Blood chemistries were normal except for a slightly elevated glucose and urea nitrogen. The serum thyroxin was normal at 4.4 µg/100 ml. Serum electrophoresis revealed a broad increase in the yG-globulins which were 19.9 g/100 ml. Serum complement activity was estimated through measurement of C3 by the radial immunodiffusion technique (23) using reagents from Hyland1 and it was normal at 163 mg/100 ml. Immuno-electrophoresis of the patient's serum revealed no paraproteins. Plasma erythropoietin, measured by the polycytemic mouse assay (24) was markedly elevated. The Schilling test (25) for vitamin B12 absorption was normal. A tuberculin purified protein derivative (PPD, intermediate strength) skin test was negative.

In vitro heme synthesis

Marrow cell cultures. The bone marrow cell cultures were prepared as previously described (21, 22). Aspirated marrow cells were placed in a sterile tube5 which contained 2 ml of NCTC-1094 with heparin (5 U/ml), penicillin (50 U/ml), and streptomycin (27 µg/ml). The cells were passed through a 10 ml pipette6 10 times to obtain adequate dispersion and were centrifuged for 10 min at 1000 g. The plasma and marrow fat, which floated to the top of the liquid, were removed. The cells were suspended in 5 ml of Hanks' balanced salt solution6 with the same concentration of heparin and antibiotics and were recentrifuged. The Hanks' solution was removed and this procedure was repeated three times. The cells were then diluted with a portion of the above NCTC-109 solution and were transferred to 35 × 20 mm tissue culture dishes.7 The culture medium consisted of 60% NCTC-109, 20% normal human plasma from a type AB donor and 20% sterile-filtered precolostrum, newborn calf serum.8 The cells were incubated at 37°C in an atmosphere of 5% CO2 and 95% air. Sheep plasma erythropoietin concentrate, 3.3 U/mg protein (Step III, lot K147 187),8 or human urinary erythropoietin concentrate, 65 U/mg protein (Belcher pool H obtained from Dr. Frederick Stohlman, Jr.) were added to the cells as indicated. 5Fe as ferric chloride was attached to transferrin by incubation with a mixture of 60% NCTC-109 and 40% human AB plasma at 37°C overnight, and 0.1–0.3 ml of this solution containing 0.4–3.2 µCi was added to the cultures.

Cyclohexanone extraction of heme. At the end of an incubation period the contents of the dishes were transferred to glass tubes. The dishes were washed with 2 ml of Bacto Hemagglutination Buffer9 which was added to the tubes and the latter were centrifuged for 5 min at 1000 g. The supernatants were discarded and the cells were washed once more with 2 ml of this buffer. The packed cells were lysed by the addition of 1.8 ml of Drabkin's solution (26), diluted to one-third of its usual concentration, and were allowed to stand overnight at 3°C. The heme was then extracted from the lysate by the method of Teale (27) as

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1 Hyland Laboratories Div., Los Angeles, Calif.
2 Falcon Plastics, Oxnard, Calif.
4 Grand Island Biological Co., Grand Island, N. Y.
5 Colorado Serum Co., Denver, Col.
6 U. S. Public Health Service Study Section on Erythropoietin.
7 Difco Laboratories, Detroit, Mich.
modified by Hrinda and Goldwasser (28). The lysate was mixed thoroughly with 0.2 ml of 2 N HCl followed by 2 ml of cyclohexanone and the tubes were centrifuged for 30 min at 2000 g. A portion of the upper phase was then counted either in an automatic gamma well-type scintillation counter (Model 4223) or an infinite thinness in a gas flow geiger counter with a background of 1.3 cpm (Model 1152). The error in counts per minute was less than 1% (95% confidence level) (29). It has been previously demonstrated that the "Fe in the upper phase is heme iron (21). In experiments where several plasmas were utilized the fraction of medium "Fe incorporated into heme was multiplied by the total iron in the medium to calculate the picograms of iron incorporated into heme. The medium iron was derived entirely from plasma and serum and was determined by the method of Ramsay (30).

**Isolation of hemoglobins and γG-globulins**

**Carboxy-hemoglobin.** The procedure of Huisman, Martin, and Dozy (31) as modified by Fantoni, Bank, and Marks (32) was utilized for the separation of human carboxy-hemoglobin. The patient's marrow cells were incubated with erythropoietin for 72 h before "Fe was added. The incubation was terminated 10 h later and the marrow cells were transferred to glass tubes and were washed five times as described above with 6 ml of Bacto Hemaaglutination Buffer. The cells were then lysed by being added to 5 ml of 0.001 M phosphate buffer, pH 7.3. Carbon monoxide was bubbled through the cell lysates for 10 min and they were dialyzed for 48 h at 3°C in 2 liters of 0.01 M phosphate buffer, pH 6.3. The dialyzed solutions were then placed on columns of CM-cellulose (0.9 X 25 cm) that had been equilibrated at pH 6.3 and washed with 200 ml of 0.01 M phosphate, pH 6.3. The hemo-globin was eluted from the columns at 0.5 ml/min with a gradient from pH 6.3 to pH 7.8 of 0.01 M phosphate buffer. The pH gradient was adjusted so that the pH increased from 6.3 to 7.0 with the first 50 ml of eluate and from 7.0 to 7.8 with the next 50 ml (32). 3 ml fractions were analyzed for pH and absorbance at 415 nm and were then evaporated on 2-inch planchets and counted in the gas flow geiger counter. This experiment was repeated with the same medium, but replicate samples were obtained for heme extraction and CM-cellulose chromatography. The column fractions which formed two principal peaks of radioactivity on CM-cellulose were combined into two pools, and each pool was concentrated to 4 ml by ultrafiltration with collodion bags. 2 mg of crude liver ferritin (supplied by Dr. Thomas Gabuzda) and 0.5 g of glucose were added to the concentrate of one peak before applying these solutions to Sephadex G-200 columns (25 X 36 cm) and eluting them with 0.01 M phosphate buffer, pH 7.3. The ferritin served to mark the void volume of the column while the glucose marked the total elution volume. 6-ml fractions were analyzed for absorbance at 280 nm and for glucose content by the Clinical Pathology Laboratory using the method of Brown (33). These fractions were then divided and 1/2 was evaporated on 2-inch planchets and counted in the gas flow geiger counter. The other one-half was pooled and concentrated to 1 ml. This was diluted with Drabkin's solution to the same volume as the replicate cell lysates. The absorbance at 410 nm was determined and the samples were then counted on 2-inch planchets. Absorbance and counts per minute were doubled to correct for the division of the specimens.

**Cyanothemoglobin.** Marrow cells that had been pre-labeled with "Fe for 21 h and then washed free of unincorporated "Fe, as described below for the measurement of erythroblast cytotoxicity, were lysed in 5 ml of DRA solution diluted to one-third its usual concentration. The lysates remained at 3°C overnight and two were then placed on CM-cellulose columns (0.9 X 25 cm) that had been equilibrated with 0.003 M phosphate buffer which contained 0.11 g/liter sodium bicarbonate, 0.006 g/liter potassium terticyanide, and 0.2 g/liter potassium cyanide. This buffer had a pH of 6.7 and conductivity of 0.5 mmho. The hemo-globin was eluted from the columns at 0.5 ml/min with a conductivity gradient of 0.5-14 mmho using sodium chloride to increase the conductivity. The conductivity gradient was adjusted so that it increased from 0.5-14 mmho over 120 ml of eluate. 4 ml fractions from the columns were tested with a conductivity meter (Type CPM 21) and were then analyzed for absorbance at 540 nm and for radioactivity in the well-type scintillation counter. The fractions with a peak at 540 nm were pooled and concentrated to 4 ml by ultrafiltration. The pools were then applied to Sephadex G-200 columns (2.5 X 30 cm) and eluted with the same buffer used to equilibrate the CM-cellulose columns. The void volume and the inner volume of the gel were previously marked with ferritin and glucose as described above. 6-ml fractions were analyzed for absorbance at 415 nm and were then evaporated on 2-inch planchets and counted in the well-type scintillation counter. The fractions with a peak at 540 nm were once again pooled and concentrated to 4 ml and were then extracted for heme with cyclohexanone along with eight replicate cell lysates that had not been subjected to chromatography.

**γG-globulins.** Plasma immunoglobulins were precipitated from 30 ml of plasma with ammonium sulfate at a final saturation of 40% (34) and were then dialyzed 18 h at 3°C in 400 vol of 0.01 M sodium phosphate buffer pH 7.3. The dialyzed solutions were placed on columns of DEAE-cellulose (1.5 X 25 cm) that had been equilibrated at 3°C with this buffer (35). The γG-globulins were eluted from the DEAE-cellulose at 0.25 ml/min with 0.01 M phosphate buffer, pH 7.3. It has previously been shown that these γG-globulin preparations were free of impurities demonstrable by immunoelctrophoresis with 1% agar gel in 0.025 M barbital buffer, pH 8.6, using goat polyvalent anti-human serum as well as rabbit antisem specific for human γG-globulins (11, 15). Immunoelctrophoresis of the γG-globulin fractions used here also revealed no evidence of any impurity. All glassware and columns were cleaned and prepared for tissue culture by the method of Paul (36).

The purified γG-globulins were concentrated to 7-8 ml by ultrafiltration with collodion bags that had a 25,000 mol wt pore size and had been soaked 24 h in 7X, 24 h in a sodium metasilicate (1 g/liter) and Calgon (0.1 g/liter) solution, and 24 h in 0.01 N HCl, before being rinsed with distilled water and stored at 3°C. The γG-globulins were then dialyzed in the collodion bags for 14 h in 200 vol of NCTC-109, with 50 U of penicillin and 5 μg of streptomycin per ml, followed by dialysis for 8 h in 50 vol of NCTC-109 with the same concentration of antibiotics. The γG-globulins were sterilized by filtration through

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* Nuclear Chicago Corp., Des Plaines, Ill.
* Schleicher & Schuell, Inc., Keene, N. H.
* Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

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S. B. Krantz, W. H. Moore, and S. D. Zaentz
Swinnex-25 filter units[14] with a 0.45 μm pore size and were stored at −80°C. 10 ml of Hanks' solution were passed through the filters prior to sterilization of the γ-G-globulins. Protein concentrations were measured by the ratio of absorbance at 260 and 280 nm.[37]

Treatment of these γ-G-globulins with “10-fold” purified rabbit antibody to immunochromically pure human 7S γ-globulins[15] was performed after 283 mg of the latter was dissolved in 5 ml Hanks' solution and dialyzed 24 h at 3°C in 200 vol of Hanks' solution.[7] The rabbit antibody solution was absorbed for 1 h at 26°C followed by 6 h at 3°C with an equal volume of packed marrow cells that had been washed six times with a total volume of 100 ml of Hanks' solution. It was then centrifuged for 30 min at 300 g and sterilized by passage through a Millipore 0.45 μm filter. Immunoelectrophoresis as described above using this preparation as antibody and normal human plasma as antigen showed precipitation of only γ-G-globulins. 12 mg of the absorbed antibody in 0.3 ml of Hanks' solution was added to 38 mg of the patient's γ-G-globulins in 0.8 ml of NCTC-109. These mixtures remained at room temperature for 1 h followed by 1 h at 3°C. They were then centrifuged in 5-ml tissue culture tubes[8] for 5 min at 600 g and the supernatants were removed. The precipitates were mixed with 1.7 ml NCTC-109 at 3°C and were recentrifuged. The supernatants were combined and were then added to the culture medium and marrow cells.

Measurement of cytotoxicity

Erythroblast cytotoxicity. Marrow cells that had been washed four times with Hanks' solution were mixed with 2.0 ml of the above 57Fe containing 15 μCi. Sheep erythropoietin was added at a final concentration of 0.05 U/ml and the cells were then incubated 21 h in 16 × 125 tissue culture tubes[9] set horizontally at 37°C in a 5% CO₂ atmosphere. 1 h before terminating the incubation 0.6 μCi of 51Cr as sodium chromium was added. At the end of an incubation the cells were washed six times with 10 ml of Hanks' solution. The supernatants were counted and the last supernatant had less than 2% of the radioactivity in the cells. The labeled marrow cells were suspended in Hanks' solution and 0.1 ml of the suspension was transferred to 16 × 125 mm tissue culture tubes to which 0.6 ml NCTC-109 and 0.4 ml fresh frozen human plasma had been added. γ-G-globulins were added as a portion of the NCTC-109. The tubes were incubated at 37°C for 25 h and were then centrifuged for 5 min at 1000 g. The supernatants were removed from the cells and both were counted in the automatic gamma dual channel well-type scintillation counter[10] with the channels set so that no 51Cr cpm were detectable in the 57Fe channel and 12% of the latter cpm were measured in the 51Cr channel. By the use of 57Fe and 51Cr standards the precise crossover of 57Fe cpm into the 51Cr channel was measured and subtracted from the apparent 51Cr cpm. The results are expressed as release index (RI) = (38):

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RI = \frac{\text{radioactivity of the supernatant}}{\text{total radioactivity}} \times 100.
\]

Lymphocyte cytotoxicity. The procedure of Sanderson[39] was modified for the use of blood lymphocytes. Lym-

[15] Abbreviations used in this paper: CE, cyclohexanone; PNH, paroxysmal nocturnal hemoglobinuria; RAHYGG, rabbit anti-human γG-globulin; RI, release index.

RESULTS

Response of patient's marrow cells to erythropoietin. When the patient's pretreatment marrow cells were incubated in an enriched medium with erythropoietin, the rate of 57Fe incorporation into heme increased threefold over a 3 day period (Table 1). Substitution of the

BBL, Division of Bioquest, Cockeysville, Md.
Upjohn Co., Kalamazoo, Mich.

Erythroblast Cytotoxicity of γ-G-Globulins in Pure Red Cell Aplasia
patient's plasma for normal plasma produced a 10-fold increase in this rate without the addition of erythropoietin which was already present in high concentration in the patient's plasma. A similar experiment was performed after the marrow cells were aspirated into Hank's solution with heparin and antibiotics and were incubated with only the addition of the patient's plasma. The rate of $^{59}$Fe incorporation into heme increased fourfold over a similar period (Table I).

Purification of hemoglobin to characterize source of $^{59}$Fe. Previous work with normal marrow cells has indicated that the $^{59}$Fe which is extracted from the cell lysate by the method of Teale (27) is heme iron (21) and that 93% of the heme radioactivity is derived from hemoglobin (44). Because of the small number of erythroblasts in the marrow of patients with red cell aplasia it is possible that other iron containing molecules might assume greater relative importance. For this reason, after incubation of the patient's pretreatment marrow with $^{59}$Fe, concomitant purification of hemo-
globin was performed along with heme extraction. Lysates of cells incubated with and without erythropoietin were chromatographed on CM-cellulose (Fig. 1). Two large peaks of radioactivity were noted after erythropoietin treatment (Fig. 1B) that were greatly reduced or not present without the addition of the hormone (Fig. 1A). In a repeat experiment replicate samples were obtained for heme extraction and and hemoglobin purification by column chromatography (Table II). The fractions which contained peak I (Fig. 1B) were pooled, concentrated, and mixed with unlabeled ferritin and glucose. This sample was then rechromatographed on Sephadex G-200 (Fig. 1C) The radioactivity of peak I was eluted with the ferritin in the void volume. Peak II has been previously identified as hemoglobin (31). The fractions which contained peak II (Fig. 1B) were pooled, concentrated, and rechromatographed on a replicate column of Sephadex G-200 (Fig. 1D). Two additional peaks of radioactivity were noted, peak 1, which was in the void volume and peak 2 which was in the volume occupied by hemoglobin (45). The specific activity of peak II did not differ from the specific activity of the heme extracted from the unpurified cell lysates with cyclohexanone (Table II).

Treatment of patient. Because the observations in this case of red cell aplasia were similar to those reported

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**TABLE I**

Effect of Erythropoietin on Heme Synthesis by Patient's Pretreatment Marrow Cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cultures</th>
<th>Time*</th>
<th>Erythropoietin</th>
<th>Medium iron in heme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no.</td>
<td>h</td>
<td>U/ml</td>
<td>$pg \times 10^4$/ nucleated cell</td>
</tr>
<tr>
<td>0.16 ml normal plasma</td>
<td>4</td>
<td>0–17</td>
<td>Not added</td>
<td>0.56±0.02</td>
</tr>
<tr>
<td>0.16 ml calf serum</td>
<td>4</td>
<td>0–17</td>
<td>0.17§</td>
<td>0.64±0.05</td>
</tr>
<tr>
<td>0.48 ml NCTC-109</td>
<td>4</td>
<td>48–65</td>
<td>Not added</td>
<td>(&lt;0.01)</td>
</tr>
<tr>
<td>0.58 ml Hanks' solution</td>
<td>4</td>
<td>48–65</td>
<td>0.17</td>
<td>1.79±0.08</td>
</tr>
<tr>
<td>0.16 ml patient's plasma</td>
<td>4</td>
<td>0–17</td>
<td>Not added</td>
<td>0.53±0.02</td>
</tr>
<tr>
<td>0.16 ml calf serum</td>
<td>4</td>
<td>0–17</td>
<td>0.17</td>
<td>0.57±0.01</td>
</tr>
<tr>
<td>0.48 ml NCTC-109</td>
<td></td>
<td></td>
<td></td>
<td>(&lt;0.01)</td>
</tr>
<tr>
<td>0.58 ml Hanks' solution</td>
<td>3, 4</td>
<td>48–65</td>
<td>Not added</td>
<td>6.08±0.18</td>
</tr>
<tr>
<td>0.40 ml patient's plasma</td>
<td>4</td>
<td>0–20</td>
<td></td>
<td>0.37±0.01</td>
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<tr>
<td>0.50 ml Hanks' solution</td>
<td>4</td>
<td>0–20</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(&lt;0.01)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>48–68</td>
<td>Not added</td>
<td>1.76±0.06</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>48–68</td>
<td>0.13</td>
<td>1.76±0.07</td>
</tr>
</tbody>
</table>

Mean values ±SEM are shown. *P values (42,43) are shown between pairs of corresponding measurements if $P <0.05$.

* Incubation period with 1.8 $\mu$Ci $^{59}$Fe.
† Cultures started with 700 nucleated cells/µl.
§ Human urinary erythropoietin.
¶ Sheep plasma erythropoietin (Step III).
‖ Mouse erythropoietin (Step III).
§§ Marrow cells aspirated into Hanks' solution with heparin (5 U/ml), penicillin (50 U/ml), and streptomycin (27 µg/ml).
in other cases that had responded to cytotoxic immuno-
suppressive drugs (10-12) the patient was treated with
cyclophosphamide and prednisone (Fig. 2). The cyclo-
phosphamide was gradually increased until marrow
toxicity was reached as indicated by a white cell count
of 1500 cells/μl. At this point the cyclophosphamide was
discontinued and 20 days later the patient began to have
terocytosis and sustain his red cell mass. He de-
veloped disseminated histoplasmosis following this treat-
ment, but this was successfully treated with amphot-
ericin-B. During this treatment repeated bone marrow
aspirations were performed and the rate of heme syn-
thesis was measured on the 3rd day of culture. 37 days
after beginning treatment a progressive increase in the
rate of heme synthesis of the patient’s marrow cells was
first noted. This occurred before a reticulocytosis was
apparent and was the earliest effect of this treatment
that was found. The low rates of heme synthesis at 113
and 126 days were probably due to insufficient marrow
since the nucleated cell counts were very low at those
times. The variation in erythropoietin concentration that
was utilized cannot account for more than a 50% vari-
tion in heme synthesis (21). The patient was phlebo-
tomized to remove excess storage iron and maintained a
marked erythroid hyperplasia throughout the remainder
of his hospital stay.

Effect of patient’s γG-globulins on heme synthesis by
marrow cells. Frequent blood samples were drawn
throughout this investigation and the patient’s γG-globu-
lins were purified from these specimens. The patient’s
pretreatment and posttreatment γG-globulins were then
available to test on his own posttreatment marrow cells.
When the patient’s γG-globulins were incubated with
his posttreatment marrow cells (Fig. 3A), or normal
marrow cells (Fig. 3B), a marked inhibition of heme
synthesis was produced by the γG-globulins obtained
prior to treatment. The inhibitory capacity of the pa-

tient’s γG-globulins obtained in the 3 wk period be-
tween discontinuation of cyclophosphamide and the on-
set of a reticulocytosis was greatly reduced.

Increasing concentrations of the patient’s pretreatment
and posttreatment γG-globulins were incubated with
normal marrow cells in vitro and the effect on the rate of
heme synthesis was determined (Fig. 4). The pre-
treatment γG-globulins at physiological concentrations
markedly inhibited the rate of heme synthesis compared
with the posttreatment γG-globulins. Similar results
have been obtained when the γG-globulins of another patient
with red cell aplasia were compared to normal γG-globu-

Production of erythroblast cytotoxicity by patient’s
γG-globulins. Normal marrow cells, labeled with 55Fe
and 51Cr, were incubated with the patient’s pretreatment
and posttreatment γG-globulins, normal γG-globulins,

Figure 1 Effect of erythropoietin on hemoglobin synthesis
by patient’s pretreatment marrow cells in vitro. Marrow
cultures were started with 1,760 nucleated cells/μl in total
volume of 0.87 ml with 60% NCTC-109, 20% normal human
plasma, and 20% newborn calf serum. Controls (A) had no
added hormone; stimulated cultures (B) had 0.6 U/ml of
human urinary erythropoietin. After 72 h of incubation
1.8 μCi of 55Fe was added and the cultures were terminated
10 h later. Carboxy-hemoglobin was then purified on CM-
cellulose (A, B). In a repeat experiment (Table II for
precise conditions) column peaks I and II from erythropoe-
tin-treated cultures (B) were concentrated. Peak I was
combined with unlabeled ferritin and glucose, and was
rechromatographed on a Sephadex G 200 (C). Peak II was
similarly chromatographed on a replicate column (D).

and all of the NCTC-109 media with which each γG-
fraction had been dialyzed prior to sterilization. In addi-
tion, the γG-globulins of a patient with paroxysmal
nocturnal hemoglobinuria (PNH) who had received ap-
proximately 10 blood transfusions per year over the
preceding 3 yr were tested at the same time. After this
incubation the cells were separated from the supernatants and the radioactivity was measured. Heme was then extracted from the cells and supernatants and its radioactivity was also measured (Fig. 5). The pretreatment γG-globulins produced a reduction of cell "Fe compared with the normal γG-globulins, the γG-globulins from the patient with PNH, or compared with the range of media against which these various γG-fractions had been dialyzed in their preparation. With the cellular release of "Fe there was an equal increase in the amount of "Fe found in the medium. A parallel loss of cell "Fe-labeled heme was produced by the patient's pretreatment γG-globulins with a concomitant, though unequal, increase in "Fe-labeled heme in the medium. The patient's γG-globulin preparations obtained in the 3 wk period following discontinuation of cyclophosphamide and the onset of reticulocytosis had a progressive disappearance of the capacity to produce this effect. By the time reticuloctyes appeared in the patient's blood the capacity of the γG-globulins to release "Fe into the medium was no longer discernable. In contrast to the release of "Fe from the cells an increased release of "Cr was not apparent under the same conditions.

It is conceivable that the patient's pretreatment γG-globulins might increase the amount of "Fe in the medium by inhibiting reutilization of "Fe normally released

Table II

Comparison of Cyclohexanone Extract (CE) with Carboxy-hemoglobin Purified by Column Chromatography

<table>
<thead>
<tr>
<th>Cultures*</th>
<th>Time†</th>
<th>Erythrocytes§</th>
<th>Cell lysate</th>
<th>CE</th>
<th>CE</th>
<th>CE</th>
<th>Peak 2%</th>
<th>Peak 2</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>no.</td>
<td>h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0–22</td>
<td>0</td>
<td>83.1±0.92</td>
<td>83.4±0.67</td>
<td>43.0±3.69</td>
<td>0.52±0.01</td>
<td>57.0±6.0</td>
<td>64.6±13.9</td>
<td>1.13±0.06**</td>
</tr>
<tr>
<td>4</td>
<td>52–74</td>
<td>+</td>
<td>83.4±0.57</td>
<td>84.1±0.88</td>
<td>108.4±20.10</td>
<td>1.29±0.29**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean values ±SEM are shown.
* Cultures started with 4200 nucleated cells/ml in a total volume of 0.77 ml with 60% NCTC-109, 20% normal human plasma, and 20% newborn calf serum.
† Incubation period with 3.2 μCi "Fe.
§ Human urinary erthropoietin (0.15 U/ml).
∥ Absorbance at 410 nm multiplied by volume (ml).
¶ From Fig. 1(D).
** P > 0.05 (4).

Figure 2  Effect of treatment of patient on heme synthesis by patient's marrow cells. Marrow cultures were begun with 240-2600 nucleated cell/ml in a total volume of 1.0 ml with 0.12-0.30 U/ml of human urinary or sheep erthropoietin. All cultures had 40% normal human plasma except those performed on days 1, 16, and 23, which had 20% normal human plasma and 20% newborn calf serum. The open bars represent cultures with 60% NCTC-109 and the closed bars represent cultures with Hanks' solution instead of NCTC-109. After 40-48 h of incubation 0.4-1.8 μCi of "Fe was added and the cultures were terminated 17-27 h later. Each bar represents the mean ±SEM of three or four cultures.

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from marrow cells in the process of incubation. However, when the sterile supernatants of five similar cultures of $^{59}$Fe-labeled cells (1060 cpm ±38.6 SEM) were added to nonlabeled marrow cells and incubated with the latter for 20 h before separation of the medium and cells, the latter contained only 15.4 cpm ±2.6 SEM. This indicated that very little of the $^{59}$Fe liberated into the medium is in a form that is reincorporated into marrow cells in a 20 h period.

**Purification of cyanomethemoglobin to characterize source of cell heme $^{59}$Fe.** Lysates of normal marrow cells labeled with $^{59}$Fe were placed on CM-cellulose columns and hemoglobin was eluted by increasing the conductivity (Fig. 6A). It has previously been shown that cyanomethemoglobin is eluted with a cyanide buffer system similar to carboxyhemoglobin (47). The bulk of the radioactivity was associated with the hemoglobin in peak II and that peak was concentrated and placed on G-200 Sephadex columns similar to those used for the purification of carboxyhemoglobin (Fig. 6B). A single hemoglobin peak of constant specific activity was obtained and no other components containing $^{59}$Fe appeared to be present. The specific activity of the $^{59}$Fe associated

**Figure 3** Effect of patient's γG-globulins on heme synthesis by patient's posttreatment marrow cells (A) or normal marrow cells (B). Marrow cultures were begun with 1,400 (A) or 700 (B) nucleated cells/μl in a total volume of 1.1 ml with 0.4 ml normal human plasma, 0.1 ml Hanks' solution and 0.6 ml NCTC-109. All cultures had 0.86 U/ml of sheep erythropoietin. γG-globulins were extracted from patient's plasmas obtained at intervals during treatment and were present at 8.0 mg/ml. After 44 h of incubation 0.9 μCi of $^{59}$Fe was added and the cultures were terminated 24 h later. Each point shows the mean of four cultures ±SEM. The shaded areas show the mean ±SEM for 20 cultures incubated with the NCTC-109 solutions against which each of the γG-globulin preparations had been dia
dyzed. These 20 cultures were incubated simultaneously with the other cultures and had the same labeling period, but were without the patient's γG-globulins.

**Figure 4** Dose-response curves of γG-globulins. Normal marrow cultures were begun with 3,000 nucleated cells per μl in a total volume of 1.1 ml with 0.4 ml normal human plasma, 0.1 ml Hanks' solution, and 0.6 ml NCTC-109 which contained the γG-globulins extracted from the patient's plasma prior to treatment (●—●) or after treatment (○—○). All cultures had 0.05 U/ml of sheep erythropoietin. After 46 h of incubation 0.9 μCi of $^{59}$Fe was added for 24 h before terminating the cultures. Each point represents the mean of four cultures ±SEM.

**Figure 5** Production of erythroblast cytotoxicity by patient's γG-globulins. Normal marrow cells, labeled with $^{59}$Fe and $^{51}$Cr, were incubated with γG-globulins (7.7 mg/ml) extracted from patient's plasmas obtained at intervals during treatment, normal γG-globulins, and the γG-globulins from a patient with paroxysmal nocturnal hemoglobinuria (PNH). Nucleated cell concentration was 1,350/μl and red cell concentration was 200,000/μl. After 25 h supernatant and cell radioactivity was measured and heme was then extracted and counted. Each point and bar is the mean of four cultures ±SEM. (□) cpm with PNH γG-globulins; (■) cpm with normal γG-globulins. The shaded areas show the mean ±SEM for 24 cultures incubated with the NCTC-109 solutions, against which each of the γG-globulin preparations had been dia
dyzed. These 24 cultures were incubated simultaneously with the other cultures for the same period, but did not have the purified γG-globulins.

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with two such peaks (323 cpm ±14.5 SEM/Asu U) or the cyclohexanone extracts of these peaks (307 cpm ±27.5 SEM/Asu U) did not differ statistically (P > 0.05) from the cyclohexanone extracts of eight replicate whole cell lysates (184 cpm ±4.22 SEM/Asu U) (42).

Incubation of patient's γG-globulins with antibody to γG-globulins. Rabbit antibody to human γG-globulins was incubated with the patient's pretreatment and posttreatment γG-globulins before the latter were added to the patient's posttreatment 56Fe-labeled marrow cells in vitro (Table III). The patient's pretreatment γG-globulins produced a 43% increase of 56Fe in the medium compared to the posttreatment γG-globulins. No effect was observed on the release of 51Cr into the medium which was equal for both sets of γG-globulins. After preincubation of the patient's γG-globulins with rabbit antibody to human γG-globulins the increased release of 56Fe into the medium was completely abolished.

Effect of patient's γG-globulins on lymphocyte cytotoxicity. In order to test the specificity of the patient's pretreatment γG-globulins for cellular cytotoxicity, lymphocytes were separated from other blood components and were labeled with 51Cr while marrow cells were labeled with 56Fe and 51Cr (Table IV). Nonlabeled red cells were added to the labeled lymphocytes to produce a concentration equal to the number of red cells in the marrow cell suspension. Incubation of 56Fe-labeled normal marrow cells with the patient's pretreatment γG-globulins resulted in a 70% increase in the release of 56Fe into the medium without any increase in the release of 51Cr. No increase in lymphocyte release of 51Cr was produced by the patient's pretreatment γG-globulins. The profound release of 51Cr by the lymphocytes treated with horse anti-human thymocyte gamma globulin indicates that increased injury of lymphocytes by the patient's γG-globulins would have been apparent if it had occurred.

### Table III

<table>
<thead>
<tr>
<th>Cultures</th>
<th>γG-globulins</th>
<th>Medium 56Fe</th>
<th>Cell 56Fe</th>
<th>Medium 51Cr</th>
<th>Cell 51Cr</th>
<th>56Fe RI</th>
<th>51Cr RI</th>
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<tr>
<td>no.</td>
<td>8.4 mg/ml</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>4</td>
<td>Pretreatment</td>
<td>3292±77</td>
<td>7538±130</td>
<td>170± 4.4</td>
<td>1925±17</td>
<td>30.4±0.76</td>
<td>9.11±0.26</td>
</tr>
<tr>
<td>4</td>
<td>Posttreatment</td>
<td>2298±26</td>
<td>8465±52</td>
<td>173± 3.3</td>
<td>1975±62</td>
<td>21.4±0.28</td>
<td>8.06±0.16</td>
</tr>
<tr>
<td>4</td>
<td>Pretreatment plus RAHγG</td>
<td>2349±69</td>
<td>8374±108</td>
<td>200± 6.4</td>
<td>1969±27</td>
<td>21.9±0.58</td>
<td>9.31±0.43</td>
</tr>
<tr>
<td>4</td>
<td>Posttreatment plus RAHγG</td>
<td>2323±68</td>
<td>8509±217</td>
<td>196±10.3</td>
<td>2006±53</td>
<td>21.4±0.53</td>
<td>8.78±0.56</td>
</tr>
</tbody>
</table>

Mean values ±SEM are shown. P values (42,43) are shown in parenthesis between corresponding RI measurements if P < 0.05.

* RAHγG*: rabbit anti-human γG-globulin.
† Patient's posttreatment marrow cells labeled with 56Fe and 51Cr were incubated for 25 h with patient's γG-globulins. Cell and supernatants were then separated and counts per minute measured. Nucleated cell concentration was 800/μl and red cell concentration was 150,000/μl.
TABLE IV
**Effect of Patient’s γG-Globulins on Erythroblast and Lymphocyte Cytotoxicity**

<table>
<thead>
<tr>
<th>Cultures</th>
<th>γG-globulins</th>
<th>Medium $\gamma$Fe</th>
<th>Medium $\gamma$Cr</th>
<th>$\gamma$Fe R1</th>
<th>$\gamma$Cr R1</th>
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</thead>
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<tr>
<td>no.</td>
<td></td>
<td>cpm</td>
<td>cpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marrow* (4)</td>
<td>Patient (pretreatment)</td>
<td>2343±25.7</td>
<td>296±3.94</td>
<td>27.2±0.19</td>
<td>9.58±0.08</td>
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<tr>
<td>Marrow (4)</td>
<td>Patient (posttreatment)</td>
<td>1349±33.4</td>
<td>295±6.30</td>
<td>15.7±0.16</td>
<td>9.76±0.13</td>
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<tr>
<td>Marrow (4)</td>
<td>Normal donor</td>
<td>1394±47.0</td>
<td>279±13.30</td>
<td>16.4±0.06</td>
<td>9.22±0.26</td>
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<tr>
<td>Lymphocytes†(4)</td>
<td>Patient (pretreatment)</td>
<td>1650±10.4</td>
<td>279±13.30</td>
<td>33.2±0.57</td>
<td>14.0±0.07</td>
</tr>
<tr>
<td>Lymphocytes (4)</td>
<td>Patient (posttreatment)</td>
<td>1642±27.6</td>
<td>279±13.30</td>
<td>33.2±0.57</td>
<td>14.0±0.07</td>
</tr>
<tr>
<td>Lymphocytes (4)</td>
<td>Normal donor</td>
<td>1717±50.2</td>
<td>279±13.30</td>
<td>33.2±0.57</td>
<td>14.0±0.07</td>
</tr>
<tr>
<td>Lymphocytes (4)</td>
<td>Horse anti-human γG-globulin</td>
<td>4226±67.2</td>
<td>279±13.30</td>
<td>33.2±0.57</td>
<td>14.0±0.07</td>
</tr>
</tbody>
</table>

Mean values ± SEM are shown. *P values (42,43) are shown in parenthesis between corresponding RI measurements if $P < 0.05$.

* Normal marrow cells were prelabeled 0-17 h with 6 μCi $\gamma$Fe and 17-19 h with 14 μCi $\gamma$Cr. Cells were then incubated for 23 h with γG-globulins before separation of cells and supernatants. Nucleated cell concentration was 400/μl and red cell concentration was 40,000/μl.

† Normal blood lymphocytes from same donor incubated for 17 h without $\gamma$Fe and then 2 h with 14 μCi $\gamma$Cr. After removing unincorporated $\gamma$Cr the lymphocytes were mixed with preincubated red cells and were incubated for 23 h with γG-globulins before separation of cells and supernatants. Nucleated cell concentration was 400/μl and red cell concentration was 33,000/μl.

**DISCUSSION**

When the marrow cells of this patient were incubated in normal plasma with erythropoietin a marked increase in the rate of incorporation of $\gamma$Fe into heme occurred. Studies presented here demonstrate that the increase in $\gamma$Fe-labeled heme represents an increase in $\gamma$Fe-labeled hemoglobin. Culture of these marrow cells with erythropoietin also resulted in an increased incorporation of $\gamma$Fe into a non-hemoglobin iron containing fraction of high molecular weight, Hrinda and Goldwasser (28) have shown that erythropoietin stimulates iron incorporation into ferritin in normal rat cells and the large molecular weight of ferritin would place it in this non-hemoglobin fraction. The precise composition of this high molecular weight fraction is not known, however, and it could include other components which contain $\gamma$Fe (28).

When the patient’s plasma was substituted for normal plasma the marrow cells also increased their incorporation of $\gamma$Fe into heme. It has previously been demonstrated that although the plasma of patients with pure red cell aplasia often contains γG-globulins that inhibit heme synthesis (11, 15, 46), the whole plasma may not be inhibitory (11), especially a small quantity (10). The precise reason for this is not known, but it may be due to a low plasma concentration of the inhibitor coupled with factors in the plasma that might overshadow or interfere with the action of the inhibitor, such as high erythropoietin levels and, possibly, anti-complementary activity (11).

The patient’s marrow cells also increased their rate of incorporation of $\gamma$Fe into heme when the cells were incubated in the patient’s own plasma that had been diluted with only a balanced salt solution. The smaller increase here, compared with the previous experiment, could be due to the absence of calf serum and NCTC-109, since the former has been shown to potentiate the effect of erythropoietin on marrow cells in vitro (22), and the latter is an enriched medium. Because the cells were not in contact with any new ingredients except small concentrations of heparin and antibiotics, the increased rate of heme synthesis in this experiment appears to be due to separation of the cells from an inhibitor within the host. In an experiment where the plasma itself was inhibitory it was shown that an increase in the concentration of the plasma in vitro prevented the increase in heme synthesis (10). In other situations such as this where the whole plasma is not inhibitory it is still possible that inhibition may occur in vivo because larger amounts of the inhibitor are in daily contact with the cells within the host (11).

When the patient’s γG-globulins were purified and added to normal marrow cells or his own posttreatment marrow cells under controlled conditions, a marked reduction in the rate of heme synthesis was produced, compared with the effect of his own γG-globulins obtained after treatment or to media that were similarly handled, but did not contain γG-globulins. These studies confirmed that the pure red cell aplasia present in this patient had many of the same characteristics that we have described in other cases (10-12, 15, 46). He was treated with cyclophosphamide and prednisone and responded like our other patients when he had not responded to prednisone alone in the past. Further investigations were then begun to elucidate the precise
mechanism by which the inhibition of heme synthesis was produced.

Incubation of $^{59}$Fe-labeled normal marrow cells or the patient's own posttreatment marrow cells with his pretreatment $\gamma$G-globulins produced a large increase in the release of $^{59}$Fe from the cells into the medium. The decrease in cellular $^{59}$Fe was directly proportional to the decrease in cellular radioactive heme. When cyanomethemoglobin was purified from the $^{59}$Fe-labeled marrow cells it was evident that the radioactive heme represented $^{59}$Fe-labeled hemoglobin. Although a portion of the $^{59}$Fe in the marrow cells was not in hemoglobin the release of $^{59}$Fe created by the patient's pretreatment $\gamma$G-globulins was directly proportional, and almost equal, to the release of labeled hemoglobin. Thus the loss of cellular $^{59}$Fe or its equivalent appearance in the medium is a measure of the release of hemoglobin from the marrow cells.

The marrow cells were also labeled with $^{51}$Cr. Since the number of red cells in the marrow suspension was 100-fold greater than the number of nucleated marrow cells the $^{51}$Cr was principally in the red cells (48) while the $^{59}$Fe was principally in erythroblasts (49, 50). Extensive autoradiographic studies have shown that over a 24 h period $^{59}$Fe is not incorporated into red cells or granulocytic cells, but only erythroblasts and reticulocytes (49, 50). $^{51}$Cr, however, penetrates almost all blood cells (48, 51, 52) and is attached nonspecifically to many proteins. The overriding predominance of the red cell concentration allows the use of this isotope for the labeling of red cells in clinical studies (48). While the patient's pretreatment $\gamma$G-globulins produced a large increase in the release of $^{59}$Fe from the cells into the medium, there was no increase in the release of $^{51}$Cr. Thus the patient's $\gamma$G-globulins were specifically affecting erythroblasts which contained most of the newly synthesized hemoglobin (53) and were not affecting the red cells which contained most of the $^{51}$Cr.

The release of hemoglobin through an action of the patient's $\gamma$G-globulins on erythroblasts is similar to the release of $^{51}$Cr-labeled proteins by lymphocytes when they are incubated with antilymphocyte antibodies (39). The latter effect represents damage to the lymphocytes (54) and has been termed lymphocyte cytotoxicity (39, 41, 55). We believe the former effect represents injury to erythroblasts and should be termed erythroblast cytotoxicity. Three additional sources of information support this conclusion. In the first place, it has already been demonstrated that specific $\gamma$-globulins can severely damage red cells (56) or lymphocytes (39, 54, 57). Secondly, hemoglobin is almost entirely retained within the red cell membrane which is the basis of all $^{59}$Fe kinetic studies on the survival of these cells (58). It is unlikely that such a large percentage of erythroblast hemoglobin could be released from these cells in vitro without severe injury to cells. Finally, Böttiger and Rausing (13), using electron microscopy, have recently shown fragmentation of cells which are probably erythroblasts in a case of red cell aplasia that still had some erythroblasts present in the marrow. While some erythroblasts may actually have been lysed by the action of the patient's $\gamma$G-globulins in vitro no direct evidence for complete lysis is yet available.

These experiments do not exclude the possibility that reticulocytes may be severely injured or lysed by the $\gamma$G-globulins of patients with red cell aplasia. It has been shown that reticulocytes still have transplantation antigens which are lost with development into erythrocytes (59), and it is conceivable that they might still have cell membrane components which are necessary for this injury or lysis to occur. Studies are underway with blood reticulocytes to determine if they are damaged by these $\gamma$G-globulins and, if so, to measure the extent of the injury compared to marrow cells.

The fact that the patient's pretreatment $\gamma$G-globulins damaged his own posttreatment erythroblasts eliminates histo-incompatibility factors that might be responsible for this cytotoxicity. Furthermore, the absence of red cell lysis and lymphocyte cytotoxicity by these same globulins indicates that they are fairly specific in their action. A similar effect was not produced by normal $\gamma$G-globulins or the $\gamma$G-globulins from a patient who received multiple transfusions over a similar period of time. Furthermore, as the patient was treated there was a progressive decrease in this specific cytotoxic factor in the 3 wk period preceding the onset of reticulocytosis. Although it did not decrease early in the treatment period, while the marrow was increasing its rate of heme synthesis, this could reflect a lack of sensitivity of our method for measuring this factor. The fact that the cytotoxicity was prevented by pretreatment with rabbit antibody to $\gamma$G-globulins and that the $\gamma$G-globulins were immunolectrophoretically pure indicates that the cytotoxicity probably resulted from an action of the patient's $\gamma$G-globulins and not a contaminant. Prior studies in two patients with pure red cell aplasia have shown that the $\gamma$M-globulin fractions did not inhibit heme synthesis by normal marrow cells in vitro (15, 46). It thus appears that this case of red cell aplasia was associated with $\gamma$G-globulins that specifically damaged erythroblasts and that this fraction might be responsible for the lack of erythroblasts in the patient's marrow.

This case represents the eighth patient that we have thoroughly studied up to the present time and the fifth patient to respond to cytotoxic immunosuppressive drugs when vitamins, prednisone, or androgens were not successful in producing a remission (15). Of the five patients who have had remissions, all had a response of
their marrow cells to erythropoietin in vitro which was greater than threefold, while all three of the patients who did not respond had a response of their marrows in vitro which was less than twofold. In all other respects these patients appeared to have the same disease (15). Thus the response to the hormone in vitro may not only be helpful in studying the pathogenesis of the disease but also in measuring its degree of severity and predicting its response to therapy. As our methods become more refined and the sensitivity to the cytotoxic factor increases, it is hoped that measurement of erythroblast cytotoxicity will provide a better method for identifying the cause of the disease and for following and guiding its treatment.

All of these patients have had an antinuclear antibody to erythroblast nuclei, although some have had antinuclear antibody to lymphocyte nuclei as well (10–12, 15, 46). The relation of this antibody to the γG-globulins that injure erythroblasts is not apparent at the present time. It is conceivable that the cytotoxic factor is identical to the antinuclear antibody and that common antigens exist in the nuclear and cytoplasmic membranes. It is also possible, however, that erythroblast cytotoxicity in red cell aplasia leads to immunization by erythroblast nuclei and that the antinuclear antibody arises as a secondary event. Further work is necessary to establish the precise relation which exists between these two factors. Although all of the studies reported here were performed with only one case of pure red cell aplasia, preliminary studies have indicated that γG-globulins which damage erythroblasts occur in other cases as well and that the cytotoxicity is dependent on a heat-labile cofactor which may be complement (6).

While these investigations are compatible with the idea that erythroblast cytotoxicity is being produced by an antibody, we have no evidence that eliminates the possibility that the cytotoxic factor is an immune complex. Further studies are necessary to characterize the mechanism of action of the γG-globulins and delineate the precise nature of the active molecule. Nevertheless, a system for measuring erythroblast cytotoxicity by γG-globulins has been developed with which it is now possible to explore immune damage to the bone marrow not only in red cell aplasia but in other "refractory anemias." It is conceivable that the mechanism which produces pure red cell aplasia exists in other conditions, but to a lesser degree, and that wider application of these techniques will produce information on another means by which anemia is produced in human beings.

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REFERENCES


