JCI The Journal of Clinical Investigation

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J Clin Invest. 1973;52(2):324-336. https://doi.org/10.1172/JCI107188.

Research Article

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Since the inhibition of heme synthesis could be the result of damage to erythroblasts, the patient's posttreatment marrow cells or normal marrow cells were labeled with ^{59}Fe and were then incubated with the patient's pretreatment, treatment, and posttreatment γG-globulins as well as normal γG-globulins. At the end of this 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 […]

Studies on Red Cell Aplasia

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

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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 ^{59}Fe 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 posttreatment marrow cells or normal marrow cells were labeled with ^{59}Fe and were then incubated with the patient's pretreatment, treatment, and posttreatment γG-globulins as well as normal γG-globulins. At the end of this 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 cytoxicity 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.

This study was presented in part at the First International Conference on Erythropoiesis, Capri, 11–13 October 1971 and at the 14th Annual Meeting of the American Society of Hematology, San Francisco, Calif., December 1971.

Received for publication 15 June 1972 and in revised form 14 September 1972.

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 magakaryocytopoiesis 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 coexistant 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 a 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 γG-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 γG-globulins inhibited heme synthesis by his own marrow cells in vitro. In addition, the patient's pretreatment γG-globulins, when incubated with his own marrow cells that had been prelabeled with ^{59}Fe, increased the release of labeled hemoglobin into the medium. When the patient was treated with cyclophosphamide and prednisone the erythroblast cytotoxicity of his γG-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 γG-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 granulocytopoiesis, and normal megakaryocytopoiesis. 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 i.m./wk and prednisone 20 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 Clinical 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 mediastium which was believed to represent an enlarged thyroid gland after visualization by an ^{131}I scan and aortogram. The blood had 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 γ-globulins which were 1.9 g/100 ml. Serum complement activity was estimated through measurement of C'3 by the radial immunodiffusion technique (23) using reagents from Hyland[1] and it was normal at 163 mg/100 ml. Immunoelectrophoresis of the patient's serum revealed no paraproteins. Plasma erythropoietin, measured by the polycythemic mouse assay (24) was markedly elevated. The Schilling test (25) for vitamin B_{12} 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 tube[2] which contained 2 ml of NCTC-109[3] with heparin (5 U/ml), penicillin (50 U/ml), and streptomycin (2.7 μg/ml). The cells were passed through a 10 ml pipette[2] 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 solution[4] 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.[2] 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.[5] The cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Sheep plasma erythropoietin concentrate, 3.3 U/mg protein (Step III, lot K147 187),[6] 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. ^{59}Fe 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 Buffer[7] 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°. The heme was then extracted from the lysate by the method of Teale (27) as

[1] Hyland Laboratories Div., Los Angeles, Calif.
[2] Falcon Plastics, Oxnard, Calif.
[3] Microbiological Associates, Inc., Bethesda, Md.
[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)[8] or at infinite thinness in a gas flow geiger counter with a background of 1.3 cpm (Model 1152).[8] The error in counts per minute was less than 1% (95% confidence level) (29). It has been previously demonstrated that the ^{59}Fe in the upper phase is heme iron (21). In experiments where several plasmas were utilized the fraction of medium ^{59}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 ^{59}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 Hemagglutination Buffer.[7] The cells were then lysed by being frozen and thawed three times in 4 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[9] (0.9 × 25 cm) that had been equilibrated at pH 6.3 and washed with 200 ml of 0.01 M phosphate, pH 6.3. The hemoglobin was eluated 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.[9] 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[10] columns (2.5 × 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.

Cyanomethemoglobin. Marrow cells that had been prelabeled with ^{59}Fe for 21 h and then washed free of unincorporated ^{59}Fe, as described below for the measurement of erythroblast cytotoxicity, were lysed in 2 ml of Drabkin's 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 × 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 terricyanide, and 0.2 g/liter potassium cyanide. This buffer had a pH of 6.7 and conductivity of 0.5 mmho. The hemoglobin 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 2d)[11] 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 × 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 540 nm and were then 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[9] (1.5 × 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 immunoelectrophoresis with 1% agar gel in 0.025 M barbital buffer, pH 8.6, using goat polyvalent anti-human serum[1] as well as rabbit antiserum[12] specific for human γG-globulins (11, 15). Immunoelectrophoresis 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[9] that had a 25,000 mol wt pore size and had been soaked 24 h in 7X,[13] 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,[7] with 50 U of penicillin and 5 μg of streptomycin per ml, followed by dialysis for 8 h in 50 vol of NCTC-109[8] with the same concentration of antibiotics. The γG-globulins were sterilized by filtration through

[8] Nuclear Chicago Corp., Des Plaines, Ill.
[9] Schleicher & Schuell, Inc., Keene, N. H.
[10] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[11] Radiometer, Copenhagen, Denmark.
[12] Calbiochem, Los Angeles, Calif.
[13] Linbro Chemical Co., New Haven, Conn.

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 γG-globulins. Protein concentrations were measured by the ratio of absorbance at 260 and 280 nm (37).

Treatment of these γG-globulins with "10-fold" purified rabbit antibody to immunochemically pure human 7S γ-globulins[12] 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 γG-globulins. 12 mg of the absorbed antibody in 0.3 ml of Hanks' solution was added to 38 mg of the patient's γG-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[2] 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 ^{59}Fe solution 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[2] set horizonally at 37°C in a 5% CO_2 atmosphere. 1 h before terminating the incubation 0.6 μCi of ^{51}Cr as sodium chromate 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. γG-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[8] with the channels set so that no ^{51}Cr cpm were detectable in the ^{59}Fe channel and 12% of the latter cpm were measured in the ^{51}Cr channel. By the use of ^{59}Fe and ^{51}Cr standards the precise crossover of ^{59}Fe cpm into the ^{51}Cr channel was measured and subtracted from the apparent ^{51}Cr cpm. The results are expressed as "release index" (RI)[15] (38):

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

Lymphocyte cytotoxicity. The precedure of Sanderson (39) was modified for the use of blood lymphocytes. Lymphocytes were purified from human peripheral blood using sterile polypropylene columns packed with cotton (40). The leukocyte-rich plasma from 120 ml of blood was centrifuged for 10 min at 250 *g*. The platelet-rich plasma was removed and the sedimented cells were resuspended in 9 ml of autologous platelet-poor plasma for transfer to three columns as described by Alford (40). Elution of the columns was performed at 37°C with 7 ml of Medium 199[16] followed by 12 ml of 5 mM EDTA. These two cell fractions were centrifuged for 10 min at 250 *g*. The supernatants were removed and the cells were then treated with 10 ml of Tris-buffered isotonic ammonium chloride (41) to lyse the red cells. This buffer was thoroughly mixed with the cell pellets at 37°C and the cell suspension was immediately centrifuged for 10 min at 250 *g*. The pellets were suspended in 2 ml of medium similar to that which contained the ^{59}Fe and were incubated in 16 × 125 mm tissue culture tubes parallel with the marrow cells that were being labeled with ^{59}Fe. After 17 h of incubation these tubes were centrifuged for 5 min at 1000 *g*. The cells were then suspended in 0.7 ml Hanks' solution containing 14 μCi of ^{51}Cr and were incubated for 2 h at 37°C along with the marrow cells that had received ^{51}Cr. At the end of this incubation the cells were washed six times with 10 ml of Hanks solution. The last supernatant had less than 3% of the radioactivity in the cells. It has previously been shown that this procedure did not increase the percentage of dead cells as indicated by trypan blue staining (41). The two lymphocyte pellets were suspended in 1 ml of Hanks' solution and were pooled. A differential cell count revealed 81% lymphocytes, 3% granulocytes, 1% platelets, and 15% red cells.

Peripheral blood cells from the same donor were washed four times with Hanks' solution and a packed cell volume equal to that of the ^{59}Fe-labeled marrow cells was transferred to a 16 × 125 mm tissue culture tube. These cells were incubated at 37°C at the same time as the lymphocytes and marrow cells in a similar medium, but without any isotope. They were then washed six times with Hanks' solution and were suspended in this solution. A portion was added to the ^{51}Cr-labeled lymphocyte suspension to give a concentration of red cells similar to that in the labeled marrow cell suspension. Portions of this ^{51}Cr-labeled lymphocyte-red cell suspension were then transferred to 16 × 125 mm tissue culture tubes which contained the same solutions as the tubes with the labeled marrow cells. Both sets of cultures were incubated together at 37°C. 1 mg of horse anti-human thymocyte γ-globulin (lot 16,138-8)[17] was added to a group of lymphocyte cultures to produce maximum lymphocyte lysis. After 23 h of incubation the tubes were centrifuged for 5 min at 1,000 *g*. The supernatants were removed from the cells and both were counted in an automatic gamma well-type scintillation counter. Statistical analyses were carried out using the Student *t* test (42) and were confirmed by nonparametric analysis (43) wherever possible.

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 ^{59}Fe incorporation into heme increased threefold over a 3 day period (Table I). Substitution of the

[14] Millipore Corp., Bedford, Mass.

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

[16] BBL, Division of Bioquest, Cockeysville, Md.

[17] Upjohn Co., Kalamazoo, Mich.

TABLE I

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

Medium	Cultures	Time*	Erythropoietin	Medium iron in heme
	no.	*h*	*U/ml*	*pg × 10⁶/ nucleated cell‡*
0.16 ml normal plasma	4	0–17	Not added	0.56±0.02
0.16 ml calf serum	4	0–17	0.17§	0.64±0.05
0.48 ml NCTC-109	4	48–65	Not added	0.64±0.04
0.58 ml Hanks' solution				(<0.01)
	4	48–65	0.17	1.79±0.08
0.16 ml patient's plasma	4	0–17	Not added	0.53±0.02
0.16 ml calf serum	4	0–17	0.17	0.57±0.01
0.48 ml NCTC-109				(<0.01)
0.58 ml Hanks' solution	3	48–65	Not added	6.08±0.18
	4	48–65	0.17	5.55±0.18
0.40 ml patient's plasma	4	0–20	Not added	0.37±0.01
0.50 ml Hanks' solution¶	4	0–20	0.13‖	0.37±0.04
				(<0.01)
	4	48–68	Not added	1.76±0.06
	4	48–68	0.13	1.76±0.07

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 μCi ^{59}Fe.

‡ Cultures started with 700 nucleated cells/μl.

§ Human urinary erythropoietin.

‖ Sheep plasma erythropoietin (Step III).

¶ Marrow cells aspirated into Hanks' solution with heparin (5 U/ml), penicillin (50 U/ml), and streptomycin (2.7 μg/ml).

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 hemoglobin 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. 1*B*) that were greatly reduced or not present without the addition of the hormone (Fig. 1*A*). In a repeat experiment replicate samples were obtained for heme extraction and for hemoglobin purification by column chromatography (Table II). The fractions which contained peak I (Fig. 1*B*) were pooled, concentrated, and mixed with unlabeled ferritin and glucose. This sample was then rechromatographed on Sephadex G-200 (Fig. 1*C*) 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. 1*B*) were pooled, concentrated, and rechromatographed on a replicate column of Sephadex G-200 (Fig. 1*D*). 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

in other cases that had responded to cytotoxic immunosuppressive drugs (10–12) the patient was treated with cyclophosphamide and prednisone (Fig. 2). The cyclophosphamide 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 reticulocytes and sustain his red cell mass. He developed disseminated histoplasmosis following this treatment, but this was successfully treated with amphotericin-B. During this treatment repeated bone marrow aspirations were performed and the rate of heme synthesis 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% variation in heme synthesis (21). The patient was phlebotomized 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-globulins 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. 3*A*), or normal marrow cells (Fig. 3*B*), a marked inhibition of heme synthesis was produced by the γG-globulins obtained prior to treatment. The inhibitory capacity of the patient's γG-globulins obtained in the 3 wk period between discontinuation of cyclophosphamide and the onset 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 pretreatment 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-globulins using normal marrow cells (46).

Production of erythroblast cytotoxicity by patient's γG-globulins. Normal marrow cells, labeled with ^{59}Fe and ^{51}Cr, were incubated with the patient's pretreatment and posttreatment γG-globulins, normal γG-globulins,

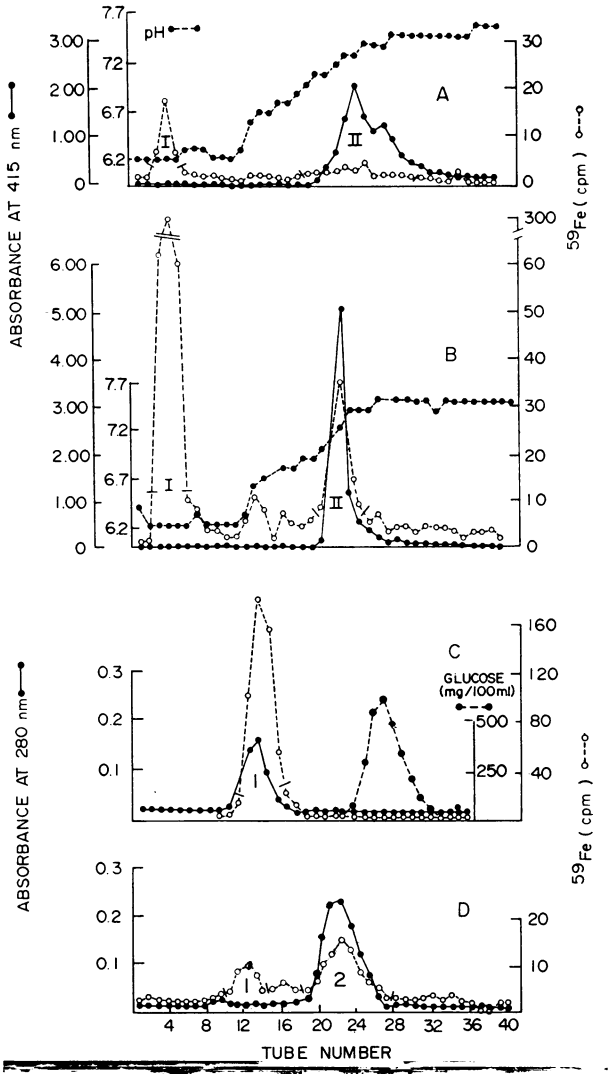

FIGURE 1 Effect of erythropoietin on hemoglobin synthesis by patient's pretreatment marrow cells in vitro. Marrow cultures were started with 1,760 nucleated cells/μl in total volume of 0.87 ml with 60% NCTC-109, 20% normal human plasma, and 20% newborn calf serum. Controls (*A*) had no added hormone; stimulated cultures (*B*) had 0.6 U/ml of human urinary erythropoietin. After 72 h of incubation 1.8 μCi of ^{59}Fe 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 erythropoietin-treated cultures (*B*) were concentrated. Peak I was combined with unlabeled ferritin and glucose, and was rechromatographed on 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 addition, the γG-globulins of a patient with paroxysmal nocturnal hemoglobinuria (PNH) who had received approximately 10 blood transfusions per year over the preceding 3 yr were tested at the same time. After this

TABLE II

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

Cultures*	Time‡	Erythropoietin§	Cell lysate	CE	CE	CE	Peak 2¶	Peak 2	Peak 2
no.	*h*		A_{410} *U*‖	A_{410} *U*	*cpm*	*cpm*/A_{410} *U*	A_{410} *U*	*cpm*	*cpm*/A_{410} *U*
3	0–22	0	85.1±0.92	83.4±0.67	43.0± 3.69	0.52±0.01			
4	52–74	+	83.4±0.57	84.1±0.88	108.4±20.10	1.29±0.26**			
2	52–74	+	101.9±1.20				57.0±6.0	64.6±13.9	1.13±0.06**

Mean values ±SEM are shown.

* Cultures started with 4200 nucleated cells/μl 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 ^{59}Fe.

§ Human urinary erythropoietin (0.15 U/ml).

‖ Absorbance at 410 nm multiplied by volume (ml).

¶ From Fig. 1(*D*).

** P >0.90 (42).

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 ^{59}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 ^{59}Fe there was an equal increase in the amount of ^{59}Fe found in the medium. A parallel loss of cell ^{59}Fe-labeled heme was produced by the patient's pretreatment γG-globulins with a concomitant, though unequal, increase in ^{59}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 reticulocytes appeared in the patient's blood the capacity of the γG-globulins to release ^{59}Fe into the medium was no longer discernable. In contrast to the release of ^{59}Fe from the cells an increased release of ^{51}Cr was not apparent under the same conditions.

It is conceivable that the patient's pretreatment γG-globulins might increase the amount of ^{59}Fe in the medium by inhibiting reutilization of ^{59}Fe normally released

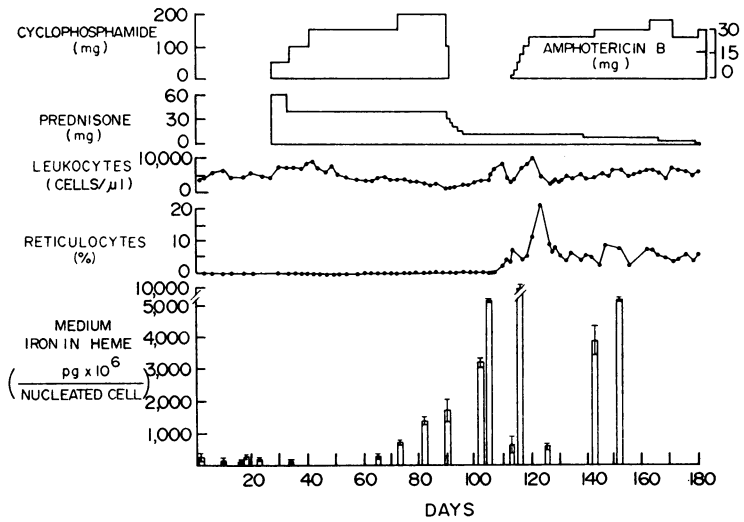

FIGURE 2 Effect of treatment of patient on heme synthesis by patient's marrow cells. Marrow cultures were begun with 240–2,600 nucleated cell/μl in a total volume of 1.0 ml with 0.12–0.30 U/ml of human urinary or sheep erythropoietin. All cultures had 40% normal human plasma except those performed on 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 ^{59}Fe was added and the cultures were terminated 17–27 h later. Each bar represents the mean ±SEM of three or four cultures.

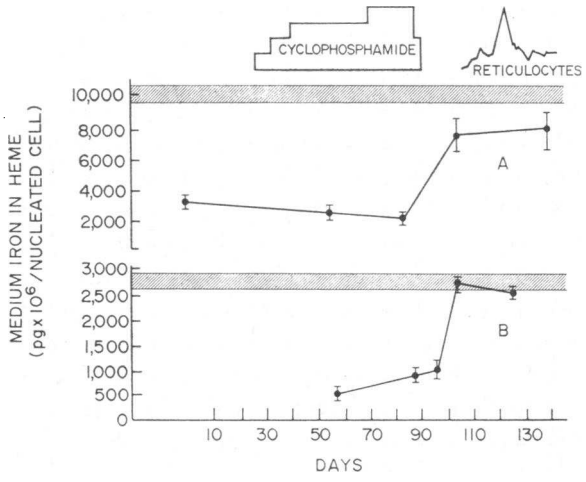

FIGURE 3 Effect of patient's γG-globulins on heme synthesis by patient's posttreatment marrow cells (*A*) or normal marrow cells (*B*). Marrow cultures were begun with 1,400 (*A*) or 700 (*B*) nucleated cells/μl in a total volume of 1.1 ml with 0.4 ml normal human plasma, 0.1 ml Hanks' solution and 0.6 ml NCTC-109. All cultures had 0.06 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 dialyzed. These 20 cultures were incubated simultaneously with the other cultures and had the same labeling period, but were without the patient's γG-globulins.

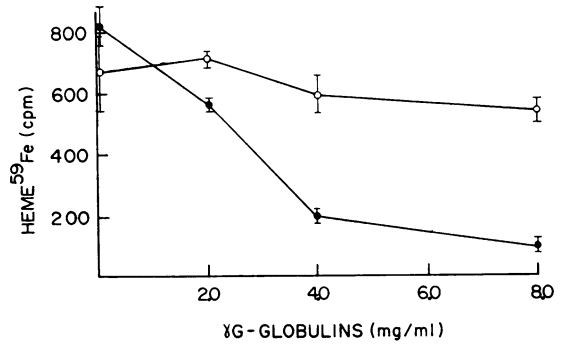

FIGURE 4 Dose-response curves of γG-globulins. Normal marrow cultures were begun with 3,000 nucleated cells per μl in a total volume of 1.1 ml with 0.4 ml normal human plasma, 0.1 ml Hanks' solution, and 0.6 ml NCTC-109 which contained the γG-globulins extracted from the patient's plasma prior to treatment (●—●) 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.

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. 6*A*). 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. 6*B*). 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

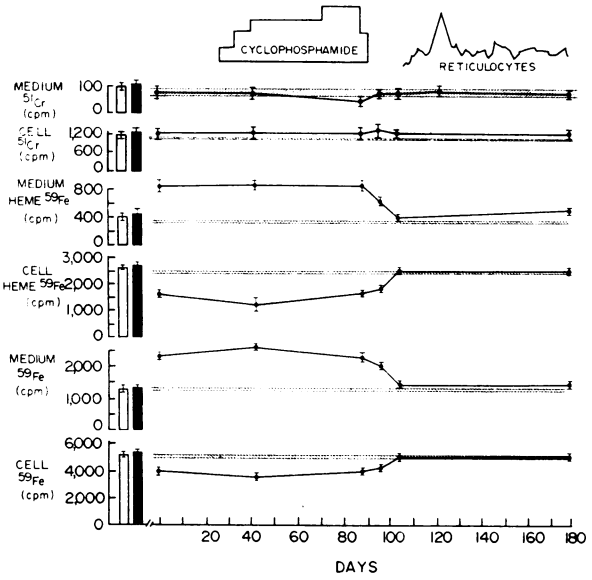

FIGURE 5 Production of erythroblast cytotoxicity by patient's γG-globulins. Normal marrow cells, labeled with ^{59}Fe and ^{51}Cr, were incubated with γG-globulins (7.7 mg/ml) extracted from patient's plasmas obtained at intervals during treatment, normal γG-globulins, and the γG-globulins from a patient 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 dialyzed. These 24 cultures were incubated simultaneously with the other cultures for the same period, but did not have the purified γG-globulins.

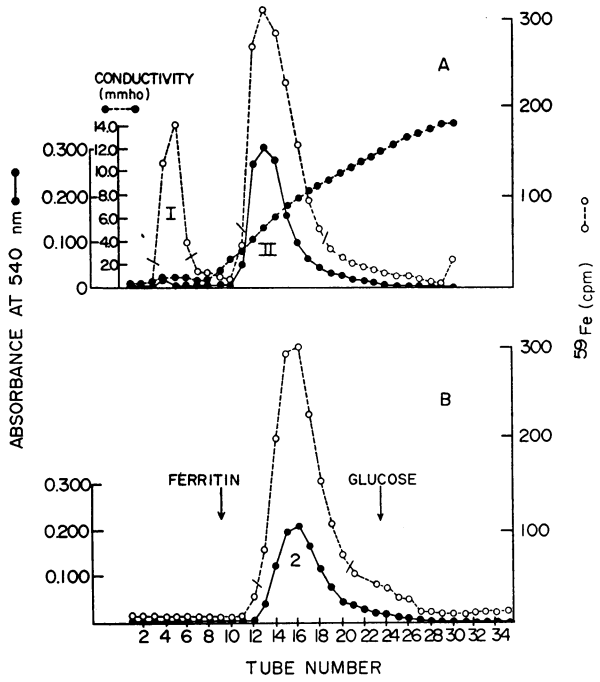

FIGURE 6 Chromatography of cyanomethemoglobin from normal marrow cells labeled with ^{59}Fe. Marrow cultures were begun with 700 nucleated cells and 250,000 red cells/μl, in a total volume of 2.0 ml, with 15 μCi ^{59}Fe and 0.05 U/ml of sheep erythropoietin. After 21 h the marrow cells were lysed and cyanomethemoglobin was purified on CM-cellulose (*A*). Peak II was rechromatographed on a G-200 Sephadex column (*B*) that had the void volume and inner gel volume previously marked with ferritin and glucose.

with two such peaks (323 cpm ±14.5 SEM/A_{540} U) or the cyclohexanone extracts of these peaks (307 cpm ±27.5 SEM/A_{540} U) did not differ statistically ($P >$ 0.05) from the cyclohexanone extracts of eight replicate whole cell lysates (184 cpm ±4.22 SEM/A_{540} 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 ^{59}Fe-labeled marrow cells in vitro (Table III). The patient's pretreatment γG-globulins produced a 43% increase of ^{59}Fe in the medium compared to the posttreatment γG-globulins. No effect was observed on the release of ^{51}Cr 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 ^{59}Fe 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 ^{51}Cr while marrow cells were labeled with ^{59}Fe and ^{51}Cr (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 ^{59}Fe-labeled normal marrow cells with the patient's pretreatment γG-globulins resulted in a 70% increase in the release of ^{59}Fe into the medium without any increase in the release of ^{51}Cr. No increase in lymphocyte release of ^{51}Cr was produced by the patient's pretreatment γG-globulins. The profound release of ^{51}Cr 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

Effect of RAHγGG on Erythroblast Cytotoxicity Produced by Patient's γG-Globulins*

Cultures‡	γG-globulins	Medium ^{59}Fe	Cell ^{59}Fe	Medium ^{51}Cr	Cell ^{51}Cr	^{59}Fe RI	^{51}Cr RI
no.	*8.4 mg/ml*	*cpm*	*cpm*	*cpm*	*cpm*		
4	Pretreatment	3292±77	7538±130	170± 4.4	1925±17	30.4±0.76 (<0.01)	9.11±0.26
4	Posttreatment	2298±26	8465± 52	173± 3.3	1975±62	21.4±0.28	8.06±0.16
4	Pretreatment plus RAHγGG	2349±69	8374±108	200± 6.4	1969±27	21.9±0.58	9.31±0.43
4	Posttreatment plus RAHγGG	2323±68	8509±217	196±10.3	2006±53	21.4±0.53	8.78±0.56

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

* RAHγGG: rabbit anti-human γG-globulin.

‡ Patient's posttreatment marrow cells labeled with ^{59}Fe and ^{51}Cr 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

Cultures	γG-globulins	Medium ^{59}Fe	Medium ^{51}Cr	^{59}Fe RI	^{51}Cr RI
no.	*11.4 mg/ml*	*cpm*	*cpm*		
Marrow* (4)	Patient (pretreatment)	2343±25.7	296± 3.94	27.2±0.19 (<0.01)	9.58±0.08
Marrow (4)	Patient (posttreatment)	1349±33.4	295± 6.30	15.7±0.16	9.76±0.13
Marrow (4)	Normal donor	1394±47.0	279±13.30	16.4±0.06	9.22±0.26
Lymphocytes‡ (4)	Patient (pretreatment)		1650±10.4		33.2 ±0.57
Lymphocytes (4)	Patient (posttreatment)		1642±27.6		33.0 ±0.07
Lymphocytes (4)	Normal donor		1717±50.2		33.6 ±0.34
Lymphocytes (4)	Horse anti-human thymocyte γ-globulin		4226±67.2		83.7 ±1.47

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 ^{59}Fe and 17–19 h with 14 μCi ^{51}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 ^{59}Fe and then 2 h with 14 μCi ^{51}Cr. After removing unincorporated ^{51}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 ^{59}Fe into heme occurred. Studies presented here demonstrate that the increase in ^{59}Fe-labeled heme represents an increase in ^{59}Fe-labeled hemoglobin. Culture of these marrow cells with erythropoietin also resulted in an increased incorporation of ^{59}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 ^{59}Fe (28).

When the patient's plasma was substituted for normal plasma the marrow cells also increased their incorporation of ^{59}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 ^{59}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 γG-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 γG-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 γG-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 γG-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 γG-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 γ-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 γG-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 γG-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 γG-globulins and, if so, to measure the extent of the injury compared to marrow cells.

The fact that the patient's pretreatment γG-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 γG-globulins or the γG-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 γG-globulins and that the γG-globulins were immunoelectrophoretically pure indicates that the cytotoxicity probably resulted from an action of the patient's γG-globulins and not a contaminant. Prior studies in two patients with pure red cell aplasia have shown that the γM-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 γG-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 compatable 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.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the fine cooperation of Dr. Francis Stein who referred this patient and supplied detailed records of his case prior to the referral.

This work has been supported in part by a grant (RR-95) from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health; by Veterans Administration Part I Research Funds and Hematology Training Grant TR No. 93; and by National Institutes of Health grant AM-15555.

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