Studies on Pure Red Cell Aplasia.

VII. Presence of Proerythroblasts and Response to Splenectomy: A Case Report

By S. Donald Zaentz, Sanford B. Krantz, and David A. Sears

An 18-yr-old female with chronic active hepatitis developed a severe anemia due to a lack of red cell production. Her bone marrow showed many large proerythroblasts but an almost complete lack of more mature erythroblasts. Incubation of the marrow cells in a normal medium with erythropoietin concentrate led to increased erythropoiesis as indicated by the development of mature erythroblasts as well as a ninefold increase in hemoglobin synthesis.

The patient's plasma was cytotoxic for erythroblasts. Following splenectomy, a remission of the disease occurred. This study indicates that in some cases the anemia associated with abundant marrow proerythroblasts and the absence of mature erythroblasts has the same pathogenesis as pure red cell aplasia and that splenectomy may be beneficial when there is a lack of response to immunosuppressive drugs.

Pure red cell aplasia (PRCA) is a condition characterized by an isolated depletion of marrow erythroblasts and blood reticulocytes. The overall marrow cellularity is generally normal, with normal granulopoiesis and megakaryocytopoiesis. The existence of an inhibitor of hemoglobin synthesis in the γG-globulin fraction of the plasma of some patients with pure red cell aplasia has been cited as evidence for an immune pathogenesis for the disease. Further evidence for such a mechanism has been provided by the demonstration of a complement-dependent factor that is cytotoxic for erythroblasts in the plasma of patients with PRCA. The presence of this cytotoxic factor in the γG-globulin fraction indicates that it is either an antibody or an immune complex. These observations have led to the use of cytotoxic immunosuppressive drugs to produce a remission of the disease when corticosteroids are ineffective.

This paper describes a patient whose marrow has almost completely devoid of mature erythroblasts but contained many proerythroblasts with some megaloblastic features. When the marrow was removed from the patient and cultured in vitro with normal plasma and erythropoietin concentrate, an increased rate of hemoglobin synthesis and an increased number of mature erythroblasts were...
noted. In addition, the patient's plasma was shown to be cytotoxic for erythroblasts. This patient failed to respond to immunosuppressive drug therapy, but splenectomy was followed by a remission of the anemia that has continued for more than 2 yr.

MATERIALS AND METHODS

The patient's marrow cells were incubated at 37°C in Falcon Plastics petri dishes with 0.16 ml normal AB plasma, 0.16 ml newborn calf serum, and 0.48 ml NCTC-109 as previously described.2-4,6,7 Four replicates were made for each incubation. Human urinary erythropoietin concentrate, 65 U per mg protein, was a gift of Dr. Frederick Stohlman, Jr. It was assayed by Dr. Stohlman in hypertransfused CF1 mice and was present at a concentration of 0.3 U/ml. Controls had no added erythropoietin concentrate. 59Fe attached to transferrin was incubated with the cells from 0 to 18 hr and from 48 to 66 hr. The 59Fe-heme, which has been shown to represent 59Fe hemoglobin,4 was then extracted. Cell concentrates and Wright-stained smears for marrow differential counts were made from unincubated quadruplicate cultures and from additional quadruplicate cultures at the end of a 44-hr incubation period. These cell cultures were transferred to tubes and were centrifuged. The supernatants were removed, and the cells were suspended in 1 ml phosphate-buffered saline before the cell concentration was measured and the marrow smears made.

Cytotoxicity for erythroblasts was measured by a modification of the two-stage technique described by Zaentz and Krantz.5,10,11 It previously has been shown that the release of 59Fe from marrow cells after treatment with PRCA plasma represents a release of 59Fe-labeled hemoglobin.4 Normal human marrow cells were first incubated with 59Fe and 51Cr and were then treated for 1 hr at 37°C with 0.46 M monosodium reduced glutathione, pH 8 (Nutritional Biochemicals, Cleveland, Ohio), before they were added to the test plasmas. All test plasmas had been incubated previously at 56°C for 1 hr. The first-stage mixtures of radioactive cells and test plasmas were incubated together for 1 hr at 22°C. The cells were then centrifuged and the test plasmas were removed for measurement of radioactivity. All first-stage supernatants had equivalent amounts of radioactivity and are not reported. The second-stage mixtures of these cells with normal plasma, as a source of complement, were then incubated for 1 hr at 37°C. The amount of radioactivity released into the second-stage supernatants was determined and expressed as a Release Index (RI)4,5

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

Statistical studies were carried out using the nonparametric Mann-Whitney U test.12 Differences were accepted as significant when \( p = 0.05 \) or less.

CASE REPORT

KL, an 18-yr-old white female, was in good health until December 1969, when she developed anorexia and jaundice. Her hematocrit and white blood cell (WBC) count were normal. A diagnosis of viral hepatitis was made on the basis of tender hepatomegaly and elevations of the serum bilirubin, alkaline phosphatase, and glutamic-pyruvate transaminase (SGPT). Following a 3-wk hospitalization, her jaundice cleared and she felt well. Jaundice recurred in March 1970. Hematocrit and WBC count were normal. SGPT was 464 mIU/ml, and serum alkaline phosphatase was 224 mIU/ml. Needle biopsy of the liver showed changes of acute viral hepatitis with prominent areas of cell necrosis and periportal inflammation. Because of the persistence of the jaundice and the elevated serum glutamic-oxaloacetic transaminase (SGOT) and SGPT, prednisone was administered beginning in May 1970. Liver function tests returned to normal, and she did well clinically.

In September 1970, she developed weakness and lightheadedness and was admitted to the Bexar County Hospital in San Antonio, Texas. She had taken no drugs except prednisone and had had no known exposure to toxic substances. Her hematocrit was 9% with less than 0.5% reticulocytes. WBC and platelet counts were normal. A bone marrow aspirate showed normal granulocytes and megakaryocytes but a virtual absence of polychromatophilic and orthochromatic erythroblasts.
PURE RED CELL APLASIA

Less than 1% proerythroblasts were present, and these had a fine chromatin and were slightly enlarged. Her plasma had a high erythropoietin content when assayed in exphypoxic polycythemic mice. Serum iron and total iron-binding capacity were 312 μg/100 ml and 332 μg/100 ml, respectively. The following tests were normal or negative: blood urea nitrogen, blood sugar, serum electrolytes, direct and indirect Coombs' with broad-spectrum antisera, serum heme pigment and haptoglobin, VDRL, lupus erythematosus preparation, latex test for rheumatoid factor, antinuclear factor, heterophile, cold agglutinin titer, serum complement (C3), antithyroglobulin, and hepatitis-associated antigen. Chest x-rays, including tomograms of the mediastinum, showed no evidence of thymoma. Liver function studies were normal. A needle biopsy of the liver showed progression of her liver disease to multifocal necrosis with portal fibrosis compatible with chronic active hepatitis.

Transfusion therapy was begun to satisfy a requirement of approximately 1 U of packed cells per wk. In November 1970, the prednisone was increased to 60 mg/day and was then gradually decreased to 30 mg/day over 3 mo. Over another 3-mo period from March to June 1971, she was given 15 mg/day of prednisone and oral androgens, fluoxymesterone, 30 mg/day for 1 mo, followed by oxymethalone, 100 mg/day for 2 mo. These trials produced no significant increase in the reticulocyte count or hematocrit and no decrease in transfusion requirement. A marrow aspirate obtained in May 1971 during androgen therapy was more cellular than the previous aspirate. Erythroblasts accounted for 2.9% of the nucleated cells, and almost all of these were proerythroblasts. Steroid therapy was discontinued in August 1971 with no change in her clinical state or liver function tests.

In November 1971, the patient was referred to the Vanderbilt University Clinical Research

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**Fig. 1.** Course of patient during treatment with cyclophosphamide and prednisone and following splenectomy.
Physical examination was unremarkable except for mild splenomegaly. Her hematocrit was 29.6%, WBC was 6000/µl, and platelet count was 291,000/µl. No reticulocytes were seen. A bone marrow particle smear showed 4.5% proerythroblasts, and the particle section was moderately hypercellular with increased granulocytopenosis and normal megakaryocytopoiesis. The marrow karyotype was normal. Immunoglobulin concentrations and C3 were within normal limits. A lupus erythematosus preparation was negative, but antinuclear antibody was present. Tests for antimitochondrial antibody and hepatitis-associated antigen were negative.

In April 1972, after the completion of the special studies described below, a trial of combined immunosuppressive therapy using cyclophosphamide and prednisone was begun (Fig. 1). The cyclophosphamide was administered orally at 100 mg/day for 20 days, then at 150 mg/day for 40 days, and finally at a dose of 200 mg/day for 14 days. Cyclophosphamide was stopped when the patient's WBC declined to 700/µl and her platelet count fell to 40,000/µl. The WBC and platelet counts rapidly returned to normal, but the reticulocyte count remained zero. A bone marrow aspirate obtained in July 1972, 2 wk after discontinuation of cyclophosphamide, was similar to the previous specimen, and the transfusion requirement remained unchanged. The prednisone dosage was then gradually decreased. Steroid therapy was discontinued in late August 1972.

Because of the failure of this immunosuppressive therapy, splenectomy was performed in September 1972. The spleen was enlarged (630 g) and showed areas of recent and healed infarction and some foci of hemotopoiesis. A wedge biopsy of the liver showed well-established postnecrotic cirrhosis with broad periportal scars and considerable hemosiderosis. Her hematocrit fell slowly following surgery, and the reticulocyte count remained zero until 7 wk postsplenectomy, when a count of 0.7% was obtained. The reticulocyte count continued to rise, and 8 wk after splenectomy nucleated red cells appeared in the blood, reaching a peak at 12 wk of over 6000/µl with a reticulocyte count of 21.5%. A bone marrow aspirate obtained at this point was hypercellular and showed extremely active erythropoiesis. The patient has subsequently maintained a normal hematocrit without transfusion, and a program of periodic phlebotomies has been instituted to relieve the transfusion siderosis.

RESULTS

When the patient's marrow cells were incubated in a normal medium with erythropoietin concentrate, the rate of hem synthesis increased from 1277 cpm ± 44.5 (SEM) to 11,368 cpm ± 382 over 3 days. Even in the absence of

<table>
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<tr>
<th>0 hr (original)</th>
<th>44 hr (in vitro)</th>
<th>Significance</th>
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<tr>
<td>Granulocytes</td>
<td>76.50 ± 2.85*</td>
<td>71.20 ± 4.13</td>
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<tr>
<td>Monocytes</td>
<td>0.08 ± 0.13</td>
<td>0.05 ± 0.05</td>
</tr>
<tr>
<td>Lymphocytes and plasma cells</td>
<td>21.50 ± 2.99</td>
<td>14.38 ± 4.21</td>
</tr>
<tr>
<td>Unclassified</td>
<td>0.08 ± 0.08</td>
<td>0.08 ± 0.13</td>
</tr>
<tr>
<td>Proerythroblasts</td>
<td>1.48 ± 0.55</td>
<td>3.25 ± 0.40</td>
</tr>
<tr>
<td>Basophilic</td>
<td>0.33 ± 0.23</td>
<td>5.83 ± 1.08</td>
</tr>
<tr>
<td>Polychromatophilic erythroblasts</td>
<td>0.03 ± 0.04</td>
<td>4.58 ± 0.68</td>
</tr>
<tr>
<td>Orthochromat</td>
<td>0</td>
<td>0.63 ± 0.53</td>
</tr>
<tr>
<td>Nucleated cells</td>
<td>525 ± 43†</td>
<td>500 ± 70</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>60,000 ± 12,247</td>
<td>35,000 ± 5,000</td>
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*Per cent ± SEM. Each sample represents the mean ± SEM from four slides with 1000 cells counted per slide.
† Cells/µl ± SEM. Each count represents the mean ± SEM from four replicate tubes.
added erythropoietin concentrate, the rate of heme synthesis increased from 966 cpm ± 116.5 to 4949 cpm ± 572.8. These increases were similar to those reported with several other marrows from patients with PRCA.²⁻⁴⁻⁶⁻⁷

Morphologic erythroid differentiation was studied in similar cultures after incubation at 37°C for 44 hr (Table I). These marrow cells were somewhat diluted by peripheral blood, as indicated by the lower concentration of proerythroblasts compared to the marrow particle smear. Significant increases in the number of proerythroblasts, basophilic erythroblasts, and polychromatophilic erythroblasts were noted. In addition, 25 orthochromatric erythroblasts were present among 4000 nucleated cells after 44 hr of incubation, while none were observed in 4000 cells prior to incubation. No change occurred in the total number of nucleated cells or the percentage of granulocytic, lymphocytic, or plasma cells. Figures 2A and 2B demonstrate the numerous enlarged proerythroblasts that were present in the patient’s Wright-stained marrow particle smear. Microscopic fields that were especially rich in these cells were selected for photography. Figure 2C shows a polychromatophilic erythroblast, and Figure 2D shows an orthochromatric erythroblast present in smears of the cultured cells after 44 hr of incubation.

Incubation of samples of the patient’s plasma, obtained in November 1971, with ⁵⁹Fe- and ⁵¹Cr-labeled normal human marrow cells that had been treated with glutathione to increase the sensitivity to complement¹³ repeatedly resulted in an increased ⁵⁹Fe release index when compared to that produced by normal plasma. A representative experiment is shown in Table 2. The increase in the ⁵¹Cr release index was not significant, indicating that the patient’s plasma did not increase the lysis of the mature red cells.⁴⁻⁵ Plasma samples obtained in April 1972 and after the patient’s September 1972 splenectomy failed to produce an increased release of ⁵⁹Fe into the supernatant. The capacity of this latter experiment to demonstrate increased erythroblast cytotoxicity if it were present was shown by the significant increase of ⁵⁹Fe release produced by the other PRCA plasma used in the previous experiment.

**DISCUSSION**

Our patient’s bone marrow had abundant proerythroblasts, but there was an almost complete absence of late erythroblasts. This morphologic appearance has been termed a maturation arrest by investigators who postulated that it was due to an inhibitor of erythropoiesis.¹⁴⁻¹⁵ Such a maturation arrest has been recorded only rarely among cases of chronic acquired pure red cell aplasia²⁻⁸,¹⁴⁻¹⁶ which is generally defined as a condition with a selective absence or hypoplasia of erythroblasts.¹⁻¹² The defect in this case could be overcome by separating the marrow cells from the patient and incubating them in a normal medium with erythropoietin concentrate. Under these conditions, a pronounced increase in the number of well-differentiated mature erythroblasts occurred. In addition, a marked increase in the rate of hemoglobin synthesis also occurred. The increase in hemoglobin synthesis has been reported in several prior cases.²⁻⁴⁻⁶⁻⁷ and the morphologic observations of the present study showed for the first time that it represented an actual increase in erythropoiesis rather than just an increased synthesis of hemoglobin by the existing erythroblasts.
Fig. 2. Patient's marrow cells before incubation in vitro (A) x 160 before reproduction and (B) x 1000 before reproduction and after incubation in vitro (C,D) x 1000 before reproduction. In the marrow particle smear, areas were present that had large numbers of enlarged proerythroblasts with a fine nuclear chromatin pattern (A,B). After 44 hr of cell culture, increased numbers of polychromatophilic erythroblasts were present (C). In addition, orthochromatic erythroblasts were observed after cell inhibition (D) when none were seen prior to incubation.
Table 2. Effect of Patient’s Plasma on Release of $^{59}$Fe and $^{51}$Cr From Normal Human Marrow Cells

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Obtained</th>
<th>Expt. No.</th>
<th>$^{59}$Fe RI*</th>
<th>$^{51}$Cr RI*</th>
<th>Significance</th>
</tr>
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<tr>
<td>DCt</td>
<td>7-27-72</td>
<td>1</td>
<td>8.86 ± 0.12</td>
<td>2.13 ± 0.07</td>
<td>—</td>
</tr>
<tr>
<td>Lløg</td>
<td>11-24-71</td>
<td>1</td>
<td>33.94 ± 0.59</td>
<td>4.38 ± 0.18</td>
<td>$p &lt; 0.02$</td>
</tr>
<tr>
<td>KL</td>
<td>11-24-71</td>
<td>1</td>
<td>16.42 ± 0.30</td>
<td>2.18 ± 0.07</td>
<td>$p &lt; 0.02$</td>
</tr>
<tr>
<td>LCt</td>
<td>5-7-73</td>
<td>2</td>
<td>10.39 ± 0.57</td>
<td>8.15 ± 0.70</td>
<td>—</td>
</tr>
<tr>
<td>Lløg</td>
<td>12-8-71</td>
<td>2</td>
<td>25.02 ± 0.98</td>
<td>12.71 ± 0.27</td>
<td>$p &lt; 0.02$</td>
</tr>
<tr>
<td>KL</td>
<td>4-5-72</td>
<td>2</td>
<td>10.86 ± 0.59</td>
<td>9.13 ± 0.66</td>
<td>$p &gt; 0.50$</td>
</tr>
<tr>
<td>KL</td>
<td>12-29-72</td>
<td>2</td>
<td>9.69 ± 1.16</td>
<td>6.81 ± 0.72</td>
<td>$p &gt; 0.50$</td>
</tr>
<tr>
<td>KL</td>
<td>4-30-73</td>
<td>2</td>
<td>9.44 ± 0.72</td>
<td>7.39 ± 0.95</td>
<td>$p &gt; 0.50$</td>
</tr>
</tbody>
</table>

*Mean ± SEM of quadruplicate samples.
†As determined for $^{59}$Fe RI.
§Normal AB donor.
||Patient with severe PRCA.
||Patient described in this report.

In addition, this patient’s plasma, like other plasmas from patients with PRCA, had a pronounced cytotoxicity for erythroblasts. We have previously shown that the cytotoxic factor was in the γG-globulin fraction and was dependent on complement for its effect. It therefore appeared to be either an antibody or an immune complex. The factor in this patient’s plasma was similarly dependent on a heat-labile co-factor, since we observed no first-stage cytotoxicity with her heat-treated plasma. It has been shown that specific antibody has the capacity to damage red blood cell, lymphocyte, or platelet membranes sufficiently to allow the release of $^{51}$Cr-labeled protein. In some cases of PRCA, antibody or immune complex that specifically damaged erythroblasts without affecting red cells or lymphocytes has been present. In this case the plasma produced a marked cytotoxicity for erythroblasts without affecting the release of $^{51}$Cr which is principally associated with the red cells.

The plasma cytotoxicity for erythroblasts and the improved erythropoiesis after separation of the marrow from the patient’s plasma indicated that this patient’s anemia was similar to the anemia of other patients with PRCA that we have studied. However, the disease was expressed as a proerythroblast maturation arrest rather than a generalized aplasia of the erythroblasts. It has been shown with mouse fetal liver erythroblasts that an increased antigen density develops upon maturation of erythroblasts, and it has been shown with human red cells that immune hemolysis is dependent on the antigen site density. Either a qualitative or quantitative difference in the antigen site density of this patient’s erythroblasts or in the cytotoxic factor itself could account for the disproportionate decrease in the more mature erythroblasts. This study does not define an inhibitor of erythroblast maturation in this patient’s plasma. While it does not exclude the possibility of such an inhibitor, it does suggest that in some cases the morphologic picture of erythroblast maturation arrest is probably due to increased destruction of mature erythroblasts by antibody or immune complex.

PRCA can remit spontaneously, and the remission that followed resection of the spleen could have been fortuitous. Many patients with PRCA have not
responded to splenectomy. However, at least four adults with this disease have had a similar response to splenectomy. In four additional cases, the administration of immunosuppressive drugs or cobalt after splenectomy resulted in a remission when there had been no response to these agents prior to splenectomy. This difference in response could be due to the severity of the disease or to other pathogenetic factors that are still unknown. The inability to detect cytotoxic activity in the plasma sample obtained in April 1972 may indicate that a remission would have occurred even without splenectomy. However, despite this loss of activity the patient’s reticulocyte count remained zero, and her transfusion requirement was unchanged until just after the procedure. Furthermore, there are both clinical and experimental observations which support the concept that the spleen may produce factors which inhibit erythropoiesis. The spleen is known to produce antibody in human beings, and splenectomy is of demonstrable value in decreasing antibody titers in autoimmune thrombocytopenic purpura. We believe that this case and those already reported present sufficient evidence for the role of splenectomy in this rare disease to recommend it be performed when available immunosuppressive drugs have not produced a remission.

The loss of cytotoxic activity after prednisone and cyclophosphamide administration, but prior to splenectomy, could reflect a decrease to a level sufficient to cause disease but insufficient to be detected by our current methods. A lack of plasma cytotoxicity for erythroblasts has been seen in other patients with PRCA. That the insensitivity of the method to low levels of this cytotoxic factor might be responsible for these results is indicated by the fact that detection of cytotoxic activity in the November 1971 plasma sample was dependent upon the use of glutathione-treated cells. No activity was found when the plasma was tested with the previously described technique which makes use of untreated cells. Furthermore, patients with autoimmune hemolytic anemia may have no antibody detectable in their plasma at a time when antibody is found on their red cells. A similar phenomenon has been described in autoimmune thrombocytopenic purpura.

This patient’s anemia began 9 mo after the onset of hepatitis, when a liver biopsy revealed chronic active hepatitis. Viral hepatitis has been associated with various hematologic abnormalities including hemolytic anemia, reactive lymphocytosis, agranulocytosis, and aplastic anemia. Several of the neutropenic patients had a maturation arrest of the granulocytic cells, and one was shown to have an IgG plasma factor which was capable of agglutinating the patient’s neutrophils. PRCA has been described in association with a case of hepatitis following exposure to halothane, with chronic hepatitis associated with a thymoma and myasthenia gravis, and transiently in two patients with chronic viral hepatitis. Hepatitis has been associated with a variety of autoimmune phenomena. Since the plasma in this case was cytotoxic for normal erythroblasts, it is unlikely that it was directed against viral antigen. It is conceivable that the virus infection that initiates hepatitis might result in complementary antibody or immune complex that affects other tissues, but the precise relation between viral hepatitis and PRCA is uncertain.
ACKNOWLEDGMENT

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