The Proliferative Capacity of Pure Red Cell Aplasia Bone Marrow Cells

L. JAY KATZ, M.D., RONALD HOFFMAN, M.D., A. KIM RITCHEY, M.D., AND NICHOLAS DAINI AK, M.D.

Hematology Section, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut

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Pure red cell aplasia (PRCA) is a heterogeneous disorder. Immunologic abnormalities have recently been uncovered suggesting that both cell-mediated and humoral immune mechanisms may be of etiological importance in PRCA. Utilizing a technique for the cloning of bone marrow erythroid precursors, we determined the in vitro proliferative capacity of erythroid cells obtained from 21 patients with PRCA. Bone marrow cells from one group of patients produced normal or increased numbers of erythroid colonies while the in vitro proliferative capacity of bone marrow cells from a second group was characterized by subnormal erythroid colony formation. Sera obtained from the former group was frequently associated with demonstrable serum inhibitors of erythropoiesis, while PRCA in the latter group was probably the consequence of intrinsic erythroid stem cell defects or pathologic cellular interactions with nonerythroid regulatory cells. This survey of a relatively large population of patients with PRCA provides evidence for the multiple causative mechanisms that can be operative in the production of PRCA.

INTRODUCTION

Pure red cell aplasia is a disorder characterized by a selective reduction in marrow erythroid elements. The marrow is normocellular and characterized by normal granulopoiesis and megakaryocytopoiesis [1]. A varied nomenclature has been applied to this disorder. In children, this clinical syndrome is referred to as Diamond-Blackfan syndrome, congenital hypoplastic anemia, or erythropoiesis imperfecta [2]. In adults, the syndrome has been called pure red cell anemia, isolated aplastic anemia, aplastic crisis, chronic erythrocytic hypoplasia, erythroblastopenia, or red cell agenesis [3]. Unfortunately, the use of these terms supplies little information concerning the pathogenesis of the red cell aplasia and makes specific therapy difficult.

Krantz and his co-workers used marrow cell culture methods to first demonstrate an inhibitor of heme synthesis and an antibody to erythroblast nuclei in the γ G globulin fraction of a patient with pure red cell aplasia [4]. These observations have supported the general concept that, in some instances, this disorder is associated with an antibody directed against marrow precursor cells.

Recently clonal assay systems that quantitate human erythroid precursor cells have been developed [5,6]. In this investigation we have used the plasma clot culture system for the growth of erythroid colonies to study the proliferative capacity of bone marrow cells from patients with PRCA.

Address reprint requests to: Ronald Hoffman, M.D., Hematology Section, Dept. of Internal Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

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marrow cells from 21 patients with pure red cell aplasia. Our observations indicate that this disorder might be categorized into two subdivisions according to the ability of bone marrow cells from these individuals to generate erythroid colonies. The marrow cells from one group of patients are characterized by the formation of normal or greater than normal numbers of erythroid colonies, while bone marrow cells from the second group were capable of generating meager numbers of colonies in response to erythropoietin. These diverse in vitro growth patterns suggest that the pathogenesis of the pure red aplasias is heterogeneous.

MATERIALS AND METHODS

Subjects

After informed consent was obtained, 21 patients with PRCA and five normal volunteers were studied (Table 1). Each patient experienced severe anemia with reticulocytopenia. The bone marrow aspirates of each patient were normocellular with a paucity of erythroblasts. There were, however, normal numbers of megakaryocytic and granulocytic elements. These bone marrow findings were felt to be consistent with a diagnosis of pure red cell aplasia. Only patient 13 has been previously reported [7]. Five subjects were diagnosed as having idiopathic acquired PRCA (A-PRCA), four as Diamond-Blackfan syndrome (DBS) in relapse, four as transient erythroid blastopenia of childhood (TEC), one as a aplastic crisis of sickle cell anemia (AC-SS), three with chronic lymphocytic leukemia (CLL and PRCA), two as drug-induced PRCA (D-PRCA), and two as preleukemic PRCA (PL-PRCA). In this last group, acute myelogenous leukemia developed four and twelve months after the diagnosis of PRCA was established.

Erythroid Cell Cultures

Approximately 0.5 ml of marrow aspirate were collected from each subject in Eagle's minimal essential medium containing 2 percent fetal calf serum and heparin. Mononuclear cells were separated over a Ficoll-Hypaque gradient [8] and washed three times in media with 2 percent fetal calf serum. Dispersed mononuclear cells were then placed in plasma clots in a final concentration of 6 × 10^5 cells per 1.1 ml in the presence and absence of 0.0, 0.5, 1.0, 2.0, and 4.0 international units per milliliter (IU/ml) of human urinary erythropoietin (Lot M-12 TaLSL, U.S. National Institutes of Health; 12.4 IU per mg of protein) and cultured according to the method of Tepperman et al. [6]. In studies searching for humoral inhibitors of erythropoiesis, 0.1 ml of patient serum was added to cultures of the patient's own bone marrow cells containing 2.0 IU of erythropoetin/ml.

After six days of incubation at 37°C in a humidified atmosphere of 3 percent CO_2 in air, plasma clots were removed from culture and fixed in gluteraldehyde to glass slides. They were then stained with benzidine and hematoxylin and the number of colony-forming unit-erythroid (CFU-E) derived colonies were scored under 100 × magnification, counting only those colonies containing between eight and 49 benzidine-positive cells. Data concerning the ability of PRCA marrow cells to form erythropoietic bursts is not included since assays of this more primitive erythropoietic stem cell were performed in only a minority of patients.

RESULTS

The clinical diagnosis and presenting laboratory features of the 21 patients with PRCA who are the subject of this study are shown in Table 1. All the patients were
### TABLE 1
Clinical Features, Erythroid Colony-Forming Capacity and Serum Erythropoietic Inhibitory Activity of 21 Patients with Pure Red Cell Aplasia

| Pt. | Clinical* Diagnosis | Age | Transfusion* Therapy | Peripheral Blood | Bone Marrow | % Serum Inhibition of Autologous Bone Marrow Colony-Forming Unit-Erythroid |
|-----|---------------------|-----|----------------------|------------------|-------------|----------------------------------|
|     | Clinical* Diagnosis | Age | Transfusion* Therapy | Hgb (g/dl) | Retic. (%) | WBC (× 10⁹/l) | Platelets (× 10⁹/dl) | Myeloid: Erythroid Ratio | No. of CFU-E/6 × 10⁴ cells* | |
|     |                     |     |                      |          |            |               |                      |                           |                           |                 |
| 1   | A-PRCA              | 34  | Yes                  | 14.2-    | 6.2-       | 25.0-         | 5:2-4:1             |                           | 65 ± 14                      | 2                |
| 2   | A-PRCA              | 56  | No                   | 3.5      | 0.0        | 2.2           | 24.5                | 8:1                        | 60 ± 7                       | 75               |
| 3   | A-PRCA              | 38  | Yes                  | 9.4      | 0.0        | 9.5           | 15.0                | 20:1                       | 130 ± 9                     | 80               |
| 4   | A-PRCA              | 7   | Yes                  | 2.9      | 0.0        | 13.8          | 15.0                | 20:1                       | 130 ± 9                     | 80               |
| 5   | A-PRCA              | 23  | Yes                  | 6.4      | 0.5        | 5.2           | Adequate            | 24:1                       | 152 ± 23                    | 0                |
| 6   | AC-SS               | 5   | Yes                  | 3.3      | 0.0        | 15.5          | Adequate            |                           |                              |                 |
| 7   | D-PRCA              | 25  | Yes                  | 8.2      | 0.2        | 15.1          | Adequate            |                           |                              |                 |
| 8   | D-PRCA              | 28  | No                   | 3.4      | 0.2        | 8.7           | Adequate            |                           |                              |                 |
| 9   | CLL-PRCA            | 78  | Yes                  | 6.8      | 0.3        | 15.5          | Adequate            |                           |                              |                 |
| 10  | DB                  | 0.8 | No                   | 3.3      | 1.8        | 11.7          | Adequate            |                           |                              |                 |
| 11  | TEC                 | 4.0 | No                   | 4.8      | 0.2        | 4.3           | Adequate            |                           |                              |                 |
| 12  | CLL-PRCA            | 74  | Yes                  | 7.5      | 0.1        | 18.0          | Adequate            |                           |                              |                 |
| 13  | CLL-PRCA            | 82  | Yes                  | 7.6      | 1.0        | 17.5          | Adequate            |                           |                              |                 |
| 14  | PL-PRCA             | 56  | Yes                  | 6.4      | 1.0        | 4.2           | Adequate            |                           |                              |                 |
| 15  | PL-PRCA             | 75  | Yes                  | 4.4      | 0.5        | 3.2           | Adequate            |                           |                              |                 |
| 16  | DB                  | 3   | No                   | 4.4      | 0.0        | 9.5           | Adequate            |                           |                              |                 |
| 17  | DB                  | 25  | Yes                  | 3.5      | 0.1        | 2.2           | Adequate            |                           |                              |                 |
| 18  | DB                  | 17  | Yes                  | 7.8      | 1.4        | 6.5           | 235.5              | 90:1                       | 10 ± 1                       | 0                |
| 19  | TEC                 | 9   | No                   | 6.9      | 0.1        | 7.0           | 35.0                | 8:1                        | 18 ± 2                       | 0                |
| 20  | TEC                 | 8   | No                   | 8.3      | 0.1        | 7.1           | 51.2                | 9:1                        | 0 ± 0                       | NA               |
| 21  | TEC                 | 6   | No                   | 6.6      | 0.1        | 5.8           | 44.0                | 11:1                       | 0 ± 0                       | NA               |

*Abbreviations used: A-PRCA—acquired pure red cell aplasia; AC-SS—aplastic crisis of sickle cell anemia; D-PRCA—drug-induced pure red cell aplasia; CLL-PRCA—chronic lymphocytic leukemia associated with pure red cell aplasia; PL-PRCA—pure red cell aplasia that terminated in acute leukemia; DB—Diamond-Blackfan anemia; TEC—transient erythroblastopenia of childhood

*Indication of exposure to blood component therapy prior to performance of studies searching for a serum inhibitor of erythropoiesis

*Each value represents the mean ± 1 standard error of the mean. All studies were carried out in quadruplicate.

*NA—not available
severely anemic and reticulocytopenic. Their bone marrow aspirations were characterized by normocellularity and normal granulocytic and megakaryocytic maturation. The most striking abnormality, however, was a virtual absence of erythroid elements except for patients 4, 19, and 20 in which there was an arrest of erythroid maturation at the proerythroblast stage. In each case there was agreement among several independent observers as to the diagnosis and identity of bone marrow cells. Patient 7's red cell aplasia was associated with oxacillin ingestion, while patient 8's aplasia was temporally related to isoniazid therapy. None of the patients were found to have thymomas after the performance of routine chest X-rays and mediastinal tomography in patients 1, 2, 3, 5, and 14.

Bone marrow cells from five hematologically normal adult donors were able to generate optimal numbers of erythroid colonies (65 ± 14 mean ± 1 standard error of the mean [SEM] erythroid colonies/6 × 10⁴ cells) in response to 2.0 IU Ep/ml. We were unable to obtain normal control marrow specimens from children since this was felt to be inappropriate by the Human Investigation Committee of the Yale University School of Medicine. Bone marrow cells from patients 1–11 were able to form in response to a similar erythropoietin dose 103 ± 24 mean ±1 SEM erythroid colonies/6 × 10⁴ cells. CFU-E derived erythroid colony formation by PRCA marrow cells was not increased by addition of greater amounts of erythropoietin. On the other hand, bone marrow cells from patients 12–21 generated only 4 ± 1 mean ± ISEM CFU-E/6 × 10⁴ cells under similar experimental conditions. The increased numbers of CFU-E derived colonies produced by bone marrow cells obtained from the first group of PRCA patients is especially striking since very few erythroid precursors were recognized on morphological examination.

Humoral inhibitors of erythropoiesis were sought by addition of patient's own serum and that of normal controls to the patient's own marrow cells. Normal control sera did not have a statistically significant effect on the number of CFU-E derived colonies generated by PRCA bone marrow cells. We have previously shown that sera from individuals with comparable degrees of anemia due to aplastic anemia actually increased colony formation [9]. This increase in colony numbers is probably due to the large amounts of erythropoietin in these specimens. Of the 15 patients with PRCA, only sera from patients 1, 2, 4, 5, and 11 markedly inhibited the ability of their own marrow cells to generate erythroid colonies in vitro. Of the five patients with demonstrable serum erythropoietic inhibitory activity, only three had received previous transfusion therapy. Sera from all these five patients also significantly inhibited CFU-E derived colony formation by normal marrow cells.

DISCUSSION

The category of PRCA in a particular patient is often determined by the age of the patient, the association with drug ingestion, or the presence of a co-existing hemolytic anemia or leukemic disorder. These diagnostic labels are often convenient but give little information concerning the pathogenetic mechanism responsible for the production of the particular PRCA. Understanding this mechanism might be useful in designing appropriate therapy for the individual patient. Using a system for the clonal growth of committed erythroid stem cells, we have attempted to determine the proliferative capacity of PRCA bone marrow cells. Regardless of the clinical setting, patients with red cell aplasia can be allocated to one of two groups on the basis of the in vitro proliferative capacity of their bone marrow cells. One group is characterized by near normal or increased CFU-E proliferation, while the second group is characterized by markedly diminished or absent CFU-E colony growth.

The erythroid stem cell compartment of patients with PRCA associated with
normal or increased marrow erythroid colony growth appear to be qualitatively and quantitatively intact, since placement in an \textit{in vitro} environment results in normal proliferation. These findings are similar to data originally reported by Krantz and colleagues, who showed that isolated marrow cells of a patient with pure red cell aplasia responded to erythropoietin \textit{in vitro} by appropriate increase in the rate of heme synthesis [4]. It has subsequently been shown that plasma from some patients with PRCA inhibited this increase in heme synthesis, whereas normal plasma does not and that the inhibitory activity resided in the IgG fraction of the plasma [4,10–12]. The red cell aplasia in patients 1–11 in our series would seem to be produced by a mechanism similar to that originally described by Krantz and his co-workers in that a circulating inhibitor of erythropoiesis apparently inhibited the normal proliferation of CFU-E \textit{in vivo} [4]. We were, however, able to demonstrate serum erythropoietic inhibitory activity directed against autologous bone marrow cells in only five of our patients. The inability to detect serum inhibitors in the other six patients may reflect the insensitivity of our assay system in detecting such factors or that the PRCA in these individuals is due to a deficiency of functionally active erythropoietin.

The pathogenesis of the PRCA in patients with deficient bone marrow erythroid colony formation is probably different from that described in the first group described. In this group the deficient \textit{in vitro} erythropoiesis may be due to a deficiency of stem cells, a qualitative abnormality of the CFU-E resulting in a defective response to erythropoietin, or the presence of cellular inhibitors (lymphocyte or macrophage) of erythropoiesis resulting in defective erythroid differentiation. When sera from these patients were examined, erythropoietic inhibitory activity was not demonstrable using allogeneic bone marrow cells as a target tissue (data not shown).

Four of our patients presented with a clinical constellation consistent with a diagnosis of transient erythroblastopenia of childhood (TEC). TEC is a form of pure red cell aplasia which occurs over a wide age range in previously normal children and spontaneously remits within a few months with only supportive therapy [13]. Koenig and co-workers have recently shown that bone marrow mononuclear cells from a child with TEC produced normal numbers of erythroid colonies when cultured in a system free of the child's own plasma and that serum from this child showed suppressive activity against erythroid colony formation by autologous and allogeneic bone marrow cells which disappeared after hematologic recovery occurred [14]. These workers have concluded that the anemia of TEC is caused by an immune mechanism that directly suppresses erythroid progenitor cell differentiation and that this suppressive activity resides in a plasma IgG fraction. Our data obtained in patient 11 is virtually identical to that presented by Koenig and is consistent with a humoral erythropoietic inhibitory factor having pathogenetic significance in TEC. The \textit{in vitro} proliferative capacity of the bone marrow cells obtained from our three remaining patients with TEC, however, was markedly deficient when compared to normal bone marrow cells. Recent evidence demonstrating suppression of erythropoiesis by an adherent bone marrow cell population in patients with chronic infections [15] suggests the possibility of a transient activation of bone marrow adherent cells following a minor infectious episode which might result in suppression of erythropoiesis in a subset of patients with TEC. Our data suggest that the pathogenesis of TEC is diverse and that this syndrome can be produced not only by immune suppression of erythropoiesis but perhaps by transient cellular suppression of erythropoiesis.

The demonstration of antibodies in the sera of patients with PRCA which are
active against erythroid progenitor cells in vitro often results in the use of immuno-suppressive agents and the reconstitution of normal red blood cell population [1]. Current techniques to detect antibodies with erythropoiesis inhibitory activity are relatively crude and insensitive. Recently Beard et al. reported four patients with PRCA in whom antibodies capable of reacting with erythroblasts were not detected but each of these patients responded clinically to either prednisone or cyclophosphamide therapy [16]. Our use of an in vitro clonal assay system for erythroid colonies to categorize two subpopulations of patients with PRCA might allow for a more rational use of cytotoxic agents in this disorder. The PRCA of those patients who have normal or increased numbers of marrow CFU-E derived colonies would appear to be secondary to a humoral inhibitor of erythropoiesis. If the PRCA of these individuals does not spontaneously resolve as in TEC and AC-SS, or resolve following discontinuation of a drug that the particular patient is ingesting, these individuals would be ideal candidates for immunosuppressive therapy.

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