Pure Red Cell Aplasia (PRCA): Response of Three Patients to Cyclophosphamide and/or Anti-lymphocyte Globulin (ALG) and Demonstration of Two Types of Serum IgG Inhibitors to Erythropoiesis

By A. Marmont, C. Peschle, M. Sanguineti, and M. Condorelli

Three cases of adult pure red cell aplasia (PRCA) are reported. All patients proved refractory to various combinations of androgens and corticosteroids. The first case, harboring a thymoma, showed a complete clinical remission following cyclophosphamide therapy. The second and third responded similarly to either a combined cyclophosphamide + antilymphocyte globulin (ALG) treatment or to ALG administration preceded by a small dosage of cyclophosphamide, which had proved ineffective when administered alone. Serum IgG inhibitors to erythropoiesis were demonstrated in all cases by means of in vivo and/or in vitro techniques. The inhibitor(s), although directed against the erythroid marrow in both the first and third patients (PRCA type A), apparently functioned as an antibody to circulating erythropoietin (Ep) in the second case (PRCA type B). The inhibitor(s) was always absent in postremission samples. Additionally, experimental models for both types of human PRCA were established in normal rodents. The present studies support the contention that adult PRCA is an autoimmune disease. The therapeutic role of cytotoxic-immunodepressive agents in PRCA patients is confirmed. It is emphasized that ALG may represent an additional therapeutic tool in cases resistant to cyclophosphamide and/or steroids. In addition, cyclophosphamide proved effective in a patient harboring a thymoma not amenable to surgery. Finally, it is postulated that IgG serum antibodies, directed against an early erythroid precursor (PRCA type A) or, more rarely, circulating Ep (PRCA type B), play a major role in the pathogenesis of the disease.

Although pure red cell aplasia (PRCA) in the adult was first described half a century ago, little more than 150 cases have been reported so far, 60 of whom harbored a thymoma. PRCA has been recently regarded as an autoimmune condition, characterized by both a favorable response to thymectomy or nonsteroidal immunodepressive agents and the presence of serum inhibitor(s) to erythropoiesis. Although the immune nature of the inhibitor(s) has been disputed, several investigators have demonstrated the inhibitory activity in the IgG serum fraction. It is of relevance that the inhibitor(s) was not detected following either successful thymectomy or immunodepressive treatment. It has been suggested that the inhibitor(s)
may be directed against either the stem cell compartment,\textsuperscript{15} an early erythroid precursor,\textsuperscript{18} or the differentiated erythroid compartment.\textsuperscript{7}

Three cases of adult PRCA who responded to immunodepressive therapy are reported. In a cyclophosphamide-resistant case (patient 3), a complete remission lasting now over 12 mo was induced by antilymphocyte globulin (ALG) therapy. Of further interest is that a patient harboring a thymoma not amenable to surgery showed a favorable response to cyclophosphamide treatment (case 1). Two types of IgG serum inhibitors to erythropoiesis were demonstrated prior to but not after immunodepressive therapy: the first one (patients 1 and 3) was apparently directed against an early erythroid precursor, the second one (patient 2) against circulating erythropoietin (Ep).

CASE REPORTS

Case 1 (Fig. 1)

A 60-yr-old male patient was admitted to the Sampierdarena Hospital in October 1970 because of severe anemia. Although a chest X-ray examination at the age of 20 had shown the presence of a mediastinal mass, the anemic condition was first recognized in March 1970. On admission, physical examination was negative, except for a mediastinal dullness and multiple, maculopapular skin lesions on both arms and legs. Laboratory examinations showed Hb, 3.5 g/100 ml; Hct, 13%; RBC, 1,250,000/cu mm; MCV, 109 cu \( \mu \); reticulocyte count, 0.0%; WBC, 6700/cu mm. The differential count was neutrophils, 67; eosinophils, 3; lymphocytes, 23; monocytes, 5; Türk's cells, 2; platelets, 400,000/cu mm. The total serum protein level was 5.80 g/100 ml (2.75 g albumin and 3.05 g globulins); no paraprotein peaks were observed. Direct and indirect Coombs' test, cold agglutinins, LE cell and latex tests were negative. Antinuclear antibodies showed moderate (++) positivity of the speckled type. Total bilirubin was 0.40 mg/100 ml, serum iron 140 \( \mu \)g/100 ml. Ham's and sucrose tests were negative.

\[ \text{Fig. 1. Clinical course of patient 1.} \]
Bone marrow aspirates, obtained twice from the sternum and once from each iliac crest, showed marked hypercellularity of the granulocytic cell line and abundant megalakaryocytes. Erythroblasts were absent. Frequent clusters of mature, lymphoid cells were also observed.

Radiographic and tomographic examinations of the chest showed a large anterior mass which protruded in the left hemithorax, with a smoothly margined character. The patient refused surgical excision of the tumor. A biopsy examination showed the typical whorls of spindle-cell thymoma. Histologic examination of a skin lesion was consistent with the pattern of Kaposi's angiosarcoma.

The patient, maintained on regular packed red cell transfusions, was initially treated unsuccessfully with 100 mg/day of both testosterone propionate and prednisolone for approximately 2 mo. In the meantime an IgG inhibitor to erythropoiesis was demonstrated in his serum. In March 1971 a course of intravenous cyclophosphamide (400 mg/day up to a total dosage of 2.8 g) was instituted and discontinued at the appearance of severe leukopenia (WBC, 1800/cu mm). Twelve days after completion of this treatment, a reticulocyte peak of 15% was observed, followed by a second, impressive peak rising up to 40%. Bone marrow examinations showed marked erythroblastic repopulation and significant depletion of the granulocytic lineage. This was associated with a distinct drop of serum iron levels and a progressive increase of Hb, Hct, and RBC values up to normality. Furthermore, although the mediastinal mass was apparently not modified, the extension of Kaposi's skin lesions diminished markedly. In May 1972 a Ham acid serum test was moderately positive (++). The patient was dismissed in the same month. During the next 3 mo, follow-up examinations showed a normal hematologic condition. Thereafter, progression of Kaposi's sarcoma induced a rapid deterioration of his general condition. The patient died at home 6 mo after dismissal. His hematologic values were normal to the end.

Case 2 (Fig. 2)

A 70-yr-old woman was admitted to the Sampierdarena Hospital on January 12, 1973 because of severe anemia, which was first recognized in 1966. Since this anemic condition proved re-
fractory to various treatments, she was maintained on regular packed red cell transfusions, which lately induced sensitization reactions.

On admission the patient was extremely pale and fatigued, but otherwise her physical examination was negative. Fluoroscopic chest examination was negative; electrocardiographic tracings were normal. Laboratory examinations showed Hb, 8 g/100 ml; Hct, 25%; RBC, 2,270,000/cu mm; peripheral reticulocytes were virtually absent; WBC, 9000/cu mm. The differential count was neutrophils, 70; with one myelocyte and three metamyelocytes; lymphocytes, 20; monocytes, 10; platelets were 85,000/cu mm. The total protein level was 5.9 g/100 ml (3.7 g albumin and 2.2 g globulin); immunoglobulin values were IgG, 1080 mg/100 ml; IgA, 265 mg/100 ml; IgM, 148 mg/100 ml. No paraprotein peaks could be detected. The LE test was negative; antinuclear and anti-IgG antibodies were absent. Both direct and indirect erythrocyte antiglobulin tests, Ham’s, and sucrose tests were negative. Osmotic fragility was in the normal range. Serum iron was 220 µg/100 ml; all other biochemical data were within normal.

A series of sternal and iliac crest examinations showed a hypercellular marrow with a hyperplastic granulocytic component and near-normal megakaryocytosis; erythroblasts were not observed on any smear. Focal lymphoid infiltration was present.

The patient was not transfused during the first 5 days. However, a precipitous fall of the Hct values down to 12% required administration of a series of red cell concentrates. Thereafter, a combined therapy including dexamethasone (8 mg/day) and cyclophosphamide (200 mg/day) was instituted, up to a total dosage of 5.2 g of the latter. At this point, the WBC fell to a low of 2100/cu mm, and the platelets, which had in the meantime reached 138,000/cu mm, to 60,000/cu mm. On the same day large reticulocytes were seen in the peripheral blood, and bone marrow preparations showed erythroblastic hyperplasia with typical erythroblastic-reticulocytic islands. The reticulocyte count and Hct values gradually rose up to 5% and 25%, respectively, but did not show any further increase. At this point she was administered equine ALG (Behringwerke, Marburg, West Germany)* by intravenous drip infusion (starting from 5 ml/day, or 250 mg ALG, up to 20 ml/day) up to a total amount of 145 ml (7250 mg) over 9 days. No side-effects were observed during this treatment, which was, however, discontinued at the appearance of moderate hives and itching. The reticulocyte count progressed to a sustained peak of 10% (350,000/cu mm) and the Hct values to 37.5%.

Two weeks after the end of this treatment, the patient, who was still in good hematologic condition and had received 1 mg of repository tetracosactrin three times on alternate days after discontinuation of dexamethasone, went suddenly into diabetic coma. She was vigorously treated with insulin and bicarbonate, and promptly recovered. However, the clinical picture of progressive hepatitis and hepatic failure supervened (serum bilirubin, 3 mg/100 ml; GOT, 200 UW%; and GPT, 130 UW%). Au antigen and antibody tests were negative. Refractory hypotension and cardiac failure were followed by death on March 19, 1973.

Case 3 (Fig. 3)

A 58-yr-old woman was admitted to the Sampierdarena Hospital on April 7, 1973 because of a severe, long-standing anemia. Splenectomy, performed in April 1972, had not been followed by improvement. In January 1973 bone marrow smears showed a 1:25/100 ratio for erythroid/myeloid precursors. A course of cyclophosphamide therapy was therefore initiated at another institution (total dosage, 3 g); however, no change of either the reticulocyte count or Hct values was observed.

On admission a very pale patient with a grayish, slaty discoloration of the skin was observed. The physical findings were normal or near normal. Fluoroscopic chest examinations were negative for mediastinal tumors. Laboratory examinations showed Hb, 6 g/100 ml; Hct, 18%; RBC, 2,480,000/cu mm; peripheral reticulocytes, absent; WBC, 8600/cu mm, of which neutrophils, 68;

This ALG preparation is a sterile and pirogen-free 5% solution in saline. Virtually 100% of proteins are immunoglobulins comprising IgG and IgT, and at least 90% are 7S immunoglobulins. Full informed consent was obtained from patients 2 and 3 prior to its administration. In both cases, the procedures of the Helsinki convention were followed.
Fig. 3. Clinical course of patient 3. No effect had been obtained with a previous course of cyclophosphamide (total dosage, 3 g).

eosinophils, 2; basophils, 1; lymphocytes, 25; monocytes, 4; platelets, 460,000/cu mm. The total serum protein was 6.2 g/100 ml (albumin, 3.15 g/100 ml and globulins, 3.05 g/100 ml) with no paraprotein peaks; single immunoglobulin fractions were within normal limits. Direct and indirect antiglobulin tests, Ham and sucrose tests, and osmotic fragility were all negative or normal. All biochemical values were within normality, except for serum iron levels, which were 280-300 μg/100 ml, with UIBC, 134 μg/100 ml and TIBC, 434 μg/100 ml.

Sternal and iliac crest aspirations showed both marked hyperplasia of myeloid and mega-karyocytic series and near absence of erythroblasts. A dissemination of small lymphocytes with some focal collections was observed.

After a 3-wk period of observation, the patient was given oral cyclophosphamide (up to 2 g over a period of 12 days). This aimed to prevent antiequine ALG immunity. Corticosteroids were not administered. No peripheral reticulocytes were apparent at the end of this treatment, and no erythroblasts were observed in the bone marrow. ALG therapy was thereafter instituted by intravenous drip infusion up to 145 ml (7.25 g of globulin) over a period of 11 days. Although no immediate side-effects were observed, moderate fever with some itching and hives supervened at the end of this treatment. Brisk reticulocytosis developed after ALG therapy (peak values, 16%) which was followed by a sustained rise of Hct values up to normality. A massive erythroblastic repopulation of the bone marrow preceded these peripheral modifications; macro-erythroblastosis was readily apparent. These erythrokinetic changes were followed by a sharp drop of serum iron levels. During ALG treatment the absolute circulating lymphocyte count fell from a pretreatment value of 3030/cu mm to a low of 390/cu mm, but rebounded after discontinuation of this therapy.

The patient was dismissed in excellent condition on June 13, 1973. She was given no maintenance therapy. Twelve months later her blood data are still completely normal. Bone marrow smears are also within normal limits. However, renewed collections of small lymphocytes are apparent.
MATERIALS AND METHODS

Ferrokinetic Measurements (Patients 1 and 3)

Evaluation of radioiron plasma clearance. Plasma samples were obtained at 10, 30, 60, and 90 min, 2, 3, and 6 hr after intravenous injection of 13 μCi 59Fe citrate. Peripheral radioiron incorporation values for evaluation of ineffective erythropoiesis were determined when reaching a plateau. Surface radioactivity was determined over sacrum, heart, liver, and spleen during a 9-day period. All these examinations were performed prior to and after remission.

Immunofluorescence Investigations (Patients 2 and 3)

Sera from patients 2 and 3, maintained at -20°C until after full remission, were gently thawed and incubated at 37°C with the fixed, intensely erythroblastic bone marrow preparations of the original donors, obtained after remission. After gentle washing in buffered saline, they were exposed to fluoresceinated (FITC) antihuman globulin sera (the same currently used for ANF determinations), washed again, and inspected under both transmitted and incident BV illumination.

Studies on Ep and Inhibitor(s) in PRCA Sera (Patients 1, 2, and 3)

Collection of serum. Heparinized or nonheparinized sterile blood was collected from all patients both prior to and after complete clinical remission. Serum and plasma samples were maintained frozen at -30°C until utilized.

Preparation of rabbit antihuman Ep serum or IgG fraction from PRCA sera. Rabbit antihuman Ep serum (anti-Ep) was obtained by a modification of the method reported by Schooley and Garcia. One milliliter of anti-Ep employed here neutralizes 125 IU of human standard B Ep (National Institute for Medical Research, London) or 12.5 IU of either mouse or sheep Ep. All PRCA sera were subjected to either DEAE-cellulose or DEAE-Sephadex column chromatography and the IgG fraction thereby separated. Standard immunodiffusion and immunoelectrophoresis techniques were employed, respectively, to quantitate the IgG fraction and establish its purity. The amount of IgG is always expressed in terms of the equivalent volume of original serum.

Preparation of test materials for assay in exhypoxic polycythemic mice. The sera from all patients were incubated with anti-Ep (1 ml of PRCA serum/0.1 ml of anti-Ep) in a water bath incubator with constant shaking at 37°C for 30 min. Goat antirabbit gamma globulin (GARGG, Antibodies Inc., Davis, Calif.) was subsequently added for an additional 15 min under the above conditions. The precipitate was thereafter discarded by centrifugation. The appropriate amount of GARGG was previously ascertained by testing against known quantities of anti-Ep.

In other experiments, the IgG serum fraction from patient 1 or 2 was similarly incubated with Ep (step I sheep plasma Ep, with a specific activity of 0.5 IU/mg of protein; Connaught Medical Research Lab., Toronto, Canada) and then with goat antihuman gamma globulin (GAHGG, Antibodies Inc., Davis, Calif.). The appropriate amount of GAHGG was previously ascertained by means of a two-step incubation: (1) known quantities of IgG (from PRCA 1 or 2) were incubated with graded amounts of GAHGG; (2) after centrifugation, the supernatant was incubated with Ep. The incubations were performed according to the above procedure. This incubation (GAHGG + IgG and then + Ep) was repeated as a control together with the experiment involving incubation of IgG + Ep and then + GAHGG.

Assay of test materials in either normal or exhypoxic polycythemic mice. CF 1 female mice weighing 20-25 g were employed. The animals were maintained on a diet of laboratory pellets and tap water ad libitum. Normal mice received test materials either intraperitoneally or subcutaneously on days 0, 1, and 2. Radioiron (0.5 μCi 59Fe citrate in sterile physiologic saline) was injected, respectively, intravenously or intraperitoneally on day 3, and 24-hr percent RBC-radioiron incorporation values thereafter determined. Blood volume was assumed to be 5% of body weight. Mice with a final hematocrit value of less than 40% were discarded. In the exhypoxic polycythemic mouse assay, the animals were rendered polycythemic by exposure to intermittent hypoxia (0.42 atmospheres of air) for 18 hr/day up to a total of approximately 220 hr. Test materials, either incubated or not as described above, were injected intraperito-
Table 1. Radioiron Turnover Studies in Patient 1

<table>
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<th>59Fe Citrate Injected On</th>
<th>11/19/1970</th>
<th>4/14/1971</th>
<th>Normal Range</th>
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<tr>
<td>Plasma iron clearance (t1/2, min)</td>
<td>320</td>
<td>66</td>
<td>80-120</td>
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<td>Plasma iron pool (mg)</td>
<td>6.15</td>
<td>3.54</td>
<td>2.4-4.0</td>
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<tr>
<td>Plasma iron turnover rate (mg/day)</td>
<td>19.2</td>
<td>53.5</td>
<td>32-52</td>
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<tr>
<td>Total RBC iron (mg)</td>
<td>814</td>
<td>979</td>
<td>2500</td>
</tr>
<tr>
<td>RBC uptake (per cent 59Fe injected)</td>
<td>1.7</td>
<td>21.8</td>
<td>75-90</td>
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</table>

neally or subcutaneously starting from day 5 posthypoxia. Radioiron was administered as in normal mice on day 7. Forty-eight-hour per cent RBC-59Fe incorporation values were thereafter determined. The blood volume was assumed to be 7% of body weight. Mice with a final hematocrit of less than 56% were discarded.

Rat or human bone marrow cultures: incubation of PRCA serum IgG with Ep. Bone marrow obtained from 150-200-g male Sprague-Dawley rats, maintained on a diet of laboratory pellets and tap water ad libitum, was incubated with Ep and/or PRCA IgG fraction according to a slight modification of the method reported by Krantz et al. The marrow from tibiae and femora was collected in NTC-109 containing 200 U heparin/ml and 100 U penicillin/ml. After suspension, the cells were passed through a stainless steel filter and centrifuged (1200 rpm for 15 min). The cell pellet was resuspended in fresh NTC-109. A nucleated cell count was taken and the volume adjusted accordingly. Each plate contained 5 x 10^6 cells in 2 ml of a 60% NTC-109, 20% fetal calf serum (precolostrum), and 20% rat plasma mixture. Added Ep (step III sheep plasma Ep) was subtracted from the fetal calf serum. The volume of IgG was subtracted from the NTC-109 volume. The cell suspensions were incubated for 48 hr at 37°C in a 5% CO2-95% air gas mixture. Rat plasma was incubated with radioiron (59Fe chloride) for 24 hr (0.5 μCi/0.1 ml). At the end of the 48-hr incubation, the plates were removed from the incubator and radioiron was added (0.5 μCi/plate). After an 18-hr incubation with iron, the radioactive heme was extracted and counted.

Studies on human marrow cultures were performed similarly, employing, however, a nucleated count of 2.5 x 10^6 cells per plate.

RESULTS

Ferrokinetic Measurements (Patients 1 and 3)

Two cases of PRCA have been previously studied by means of ferrokinetic measurements prior to and after remission. The present studies in patients 1 and 3 confirm the complete absence of 59Fe incorporation by the bone marrow prior to but not after immunosuppressive therapy (Tables 1 and 2). Although peak values for radioiron incorporation during clinical remission were slightly inferior to the normal range, ineffective erythropoiesis and/or excessive hemolysis may be ruled out. Thus, an expansion of the deposit iron pool should be postulated. This is also in line with the sharp drop of radioiron plasma clearance after immunosuppressive therapy.

Table 2. Radioiron Turnover Studies in Patient 3

<table>
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</thead>
<tbody>
<tr>
<td>Plasma iron clearance (t1/2, min)</td>
<td>855</td>
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<td>80-120</td>
</tr>
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<td>Plasma iron pool (mg)</td>
<td>7.98</td>
<td>5.3</td>
<td>2.4-4.0</td>
</tr>
<tr>
<td>Plasma iron turnover rate (mg/day)</td>
<td>9.32</td>
<td>72.4</td>
<td>32-52</td>
</tr>
<tr>
<td>Total RBC iron (mg)</td>
<td>—</td>
<td>1447</td>
<td>2500</td>
</tr>
<tr>
<td>RBC uptake (per cent 59Fe injected)</td>
<td>—</td>
<td>36</td>
<td>75-90</td>
</tr>
</tbody>
</table>
Immunofluorescence Investigations (Patients 2 and 3)

In patients 2 and 3 no fluorescence could be demonstrated at the level of the erythroblasts, which appeared as “black holes” on the preparation. Conversely, the slight, aspecific fluorescence of both eosinophilic and, to a lesser extent, neutrophilic granules could be observed. Incubation of PRCA erythroblastic marrow with active SLE serum showed strong nuclear fluorescence of the homogenous and marginal types, affecting all cells of the bone marrow and including erythroblasts.

Studies on Ep and Inhibitor(s) in PRCA Sera (Patients 1, 2, and 3)

As indicated in Fig. 4, Ep levels were elevated in patients 1 and 2 (Hct, 22% and 18%, respectively), or barely detectable in patient 2 (Hct, 21%). Also of interest is that the activity was fully neutralized following incubation of serum with anti-Ep and GARGG. Although not presented here, further experiments indicated that incubation of PRCA serum Ep (cases 1 and 3) with neuraminidase induced complete neutralization of Ep. Additionally, dose-response regression lines following injection of purified serum PRCA Ep were strictly parallel to those of Ep standard B. Thus, it is apparent that, in all cases, the Ep molecule in serum showed normal biologic, immunologic, and chemical properties. During ALG therapy, however, Ep activity was elevated in the serum of patient 2 (peak values, 2.1 IU/ml; Hct value, 26%). This activity was also fully neutralized following incubation with anti-Ep and GARGG.

Figure 5 shows that, in human bone marrow cultures, the IgG fraction from the serum of patients 1, 2, and 3 exerted a significant inhibitory effect on the erythropoietic response induced by Ep. It is noteworthy that postremission serum IgG did not exert an inhibitory action. As indicated in Fig. 6, the serum...
Fig. 6. Effect in normal mice of the IgG fraction purified from the serum of either a normal subject (NORMAL), a patient with systemic lupus erythematosus (LE) without anemia, PRCA patient 1 (PRCA), or a rabbit producing antihuman Ep (A-Ep) on 24-hr per cent RBC-59Fe incorporation values (mean ± SEM). The amount of IgG injected in each mouse was equivalent to 0.2 ml of original serum per day x 3. **p < 0.01 when compared with other groups.

IgG fraction from patient 1 also induced a significant decrease of the erythropoietic activity in normal mice. Similar results were obtained with the IgG fraction from patient 2: saline-injected controls, 23.14 ± 1.71 (24-hr per cent RBC-59Fe incorporation values ± SEM), PRCA II serum IgG (0.1 ml/day), 7.11 ± 0.90. However, no inhibitory effect was apparent in normal mice injected with appropriate amounts (0.1–0.3 ml/day) of the IgG serum fraction from patient 3. It is therefore postulated that, in some PRCA cases, the IgG inhibitor is species-specific.

Figure 7 indicates that in exhypoxic polycythemic mice the IgG fraction from patients 1 and 2 exerted a significant inhibitory effect on the wave of erythropoiesis elicited by a simultaneous injection of Ep. Furthermore, prior incubation of PRCA I IgG with Ep and subsequently with GARGG led to full resto-
ration of the Ep activity, as evaluated in these mice. This clearly indicates that the Ep molecule did not interact with the IgG fraction, which does not constitute, therefore, an antibody to Ep. Comparable results have been described in previous reports.\textsuperscript{16,18} Since this inhibitor is also effective in marrow cultures, it can be excluded that it interacts with the Ep-generating organs. By implication, it is concluded that the inhibitor acts at the marrow level. It is noteworthy that a similar, two-step incubation of PRCA serum IgG from patient 2 with Ep and GARGG led to complete neutralization of the Ep activity. These results are identical to those observed following incubation of Ep with rabbit anti-Ep and GARGG. It is therefore suggested that this IgG autoantibody interacted with circulating Ep, thereby functioning as an anti-Ep molecule. Further support for this contention derives from the near absence of Ep activity in the serum of this patient prior to but not during immunosuppressive therapy, in the presence of low hematocrit values.

Figures 8 and 9 show two experimental models for human PRCA. In the first one (Fig. 8), prolonged injection of microdoses of serum IgG from patient 1 induced a sustained inhibition of the erythropoietic activity, associated with both a progressive drop of the hematocrit values and an inverse rise of Ep serum levels. Thus, at the end of the experiment, an experimental model for the PRCA condition of patients 1 and 3 was established. As shown in Fig. 9, a rabbit immunized with human urinary Ep, producing anti-Ep to both homologous and heterologous Ep, showed a sharp drop of the hematocrit values following a booster injection of human Ep. This was correlated with disappearance of erythroid precursors in bone marrow smears. No Ep was detected in the serum. It is postulated that this experimental PRCA was mediated by production of anti-Ep neutralizing endogenous rabbit Ep. Further support for this contention derives from a subsequent increase of the Ep serum levels, cor-
Fig. 10. Effect in exhypoxic polycythemic mice of Step I Ep (0.2 IU), administered either alone or with anti-Ep serum (0.2 ml), or serum IgG from patient 1 (amount equivalent to 0.2 ml of original serum), as evaluated on the basis of 48-hr per cent RBC-59Fe incorporation values (mean ± SEM). Ep was administered either simultaneously, or 12 or 24 hr before anti-Ep serum or IgG. *p < 0.01 when compared with corresponding Ep + anti-Ep values.

related inversely to a drop of anti-Ep titers and directly to both a reticulocytosis and a rise of the hematocrit values. Thus, an experimental model for PRCA of patient 2 was established.

Figure 10 shows that in exhypoxic polycythemic mice the IgG serum fraction from PRCA 1 exerted a significant inhibitory effect on the wave of erythropoiesis evoked by Ep injected simultaneously, or 12 or 24 hr earlier. In this regard, the half-life of heterologous gamma globulin in the mouse is 5 days.

DISCUSSION

All three patients showed a typical PRCA condition, associated in case 1 with a spindle-cell thymoma lasting for at least 40 yr. Similar long intervals between demonstration of the thymic tumor and onset of the anemia have been recorded previously. Although it has been suggested that a humoral inhibitor might be secreted by the thymoma, doubt has been cast on this hypothesis. A disruption of immunologic homeostasis, perhaps mediated by a loss of controlling functions of T lymphocytes, as postulated by Allison, appears more likely. The association of thymoma with Kaposi’s sarcoma is an example of the coexistence of primary tumors, again perhaps as a consequence of defective immunosurveillance.

A focal and/or disseminated infiltration of small, mature lymphocytes in PRCA marrow, previously mentioned in scattered reports, has been observed in all cases reported here. Furthermore, germinal centers have been observed on histologic sections in idiopathic PRCA. This bone marrow lymphocytosis in PRCA marrows is susceptible to more than one interpretation. The lymphocytes may be regarded as committed stem cells, which are present in large number due to impeded erythropoiesis. However, evidence has been presented against identification of human bone marrow lymphocytes as committed hematopoietic stem cells. On the other hand, similar collections of lymphocytes have been observed in chronic cold agglutinin anemia, as a possible indication of an immunoproliferative disease, in the idiopathic warm antibody variety and more generally in organs (thyroid, muscle, liver) affected by immunologically mediated diseases.

Serum IgG inhibitors to erythropoiesis were demonstrated by means of in vivo and/or in vitro techniques in all three patients prior to but not after immunodepressive therapy. In case 3, however, assay of the inhibitor in rodents, both in vivo (normal or polycythemic mice) and in vitro (rat marrow cultures), yielded negative results. Thus, it is apparent that consistent demon-
stration of the inhibitory activity in the IgG serum fraction requires studies in human bone marrow cultures.

Although the inhibitor is usually directed against the bone marrow, it apparently constitutes an antibody to circulating Ep in rare cases of PRCA. In this last regard, an anti-Ep molecule has been demonstrated by our group in the serum of only one out of five patients examined so far. Thus, two types of adult PRCA have been apparently demonstrated. The first, and most usual, one is characterized by both elevated levels of serum Ep and an IgG serum inhibitor against the erythroid marrow (type A), the second one by barely detectable levels of circulating Ep and an anti-Ep serum IgG (type B). In regard to PRCA type A, the mechanism of action of the inhibitor is not yet elucidated. Since the IgG serum fraction of case 1 exerted an inhibitory effect on the wave of erythropoiesis evoked in polycythemic mice by Ep injected 12 or 24 hr earlier, a selective action of the inhibitor at the level of the Ep-responsive cells seems unlikely. Therefore, in view of the negative immunofluorescence studies in patient 3, the possibility should be considered that the inhibitor interacted with an early erythroid precursor. This postulate is in line with the complete absence of recognizable erythroid precursors in marrow smears of these patients. However, it is of relevance that an additional inhibitory effect of PRCA IgG on the differentiated erythroid compartment ("erythroblast cytotoxicity") has been demonstrated by Krantz et al.

Strong evidence supports the contention that the inhibitors, whether of type A or B, are autoantibodies playing a major role in the pathogenesis of PRCA. Thus, the inhibitory activity is confined to the IgG serum fraction. Furthermore, it can be demonstrated before but not after remission induced by immunosuppressive therapy. In addition, experimental models for both types of human PRCA have been established in rodents. Thus, the basic criteria required to assess the pathogenetic role of these inhibitors are satisfied.

The striking remissions obtained by means of an alkylating agent and/or ALG lend further support to an autoimmune mechanism in PRCA and militate in favor of this type of therapy when the more conventional androgen-cortico-steroid treatment has failed. In addition, according to our results, cyclophosphamide should be administered in PRCA cases associated with a thymoma not amenable to surgery.

Initiation of ALG therapy in these patients was suggested by the unpredictable reactivity of the myeloid bone marrow to cyclophosphamide, the lack of response to this agent in case 3, and a former clinical trial by Krantz. Although the therapeutic role of ALG was uncertain in case 2, it was clearly demonstrated in patient 3: the latter patient, who proved fully resistant to a former course of cyclophosphamide (3 g), responded dramatically to the sequential administration of a lower amount of cyclophosphamide (2 g) followed by 7.25 g of ALG.

The chief objections to the administration of ALG in humoral autoimmune diseases are based on its fundamental effect on T lymphocytes, its rumored enhancing effect on oncogenesis, and its "toxic" side-effects. However, although ALG is fundamentally an antithymocyte serum, no difference in uptake of ALG could be detected between T and B lymphocyte populations, so that
differentiated immunofluorescent reactions could be obtained only after specific absorptions. In addition, ALG exerts a strong therapeutic effect in humorally mediated autoimmune diseases, including refractory ITP relapsing after splenectomy. Although the greatly increased risk of de novo oncogenesis in immunosuppressed-transplanted patients is well established, considerably fewer malignancies have been reported in patients similarly immunosuppressed for autoimmune diseases. Nevertheless, it has been demonstrated that ALG does not play a greater and/or different role in the breakdown of hypothetic immune mechanisms against neoplasia than conventional cytotoxic agents. Finally, early ALG toxicity, apparently due to the liberation of kinins, has been successfully eliminated in the more refined recent preparations.

In conclusion, the present studies support the contention that adult PRCA is a humorally mediated autoimmune disease. Immunosuppressive treatment, whether with cyclophosphamide alone or in combination with ALG, induced a complete remission in two corticoid-refractory cases and in one patient with a thymoma not amenable to surgery. It is proposed that IgG serum autoantibodies, usually directed against the erythroid marrow or rarely against circulating Ep (PRCA type A or B, respectively), play a major role in the pathogenesis of this disease.

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