

## Pure Red Cell Aplasia and Thymoma: Loss of Serum Inhibitor of Erythropoiesis Following Thymectomy

By HAMID AL-MONDHIRY, ESMAIL D. ZANJANI, MORTON SPIVACK,  
RALPH ZALUSKY, AND ALBERT S. GORDON

A 32-yr-old black female with pure red cell aplasia and thymoma showed complete hematologic remission after thymectomy. Bioassay of preoperative serum and urine in exhypoxic polycythemic mice revealed the presence of significant levels of erythropoiesis-stimulating factor (ESF or erythropoietin). Antierythropoietin completely neutralized the erythropoietic effect of these samples. Utilizing both normal and exhypoxic polycythemic

mice, the preoperative serum and urine were also found to contain a potent inhibitor of erythropoiesis. However, no such activity was demonstrated in saline extracts of the tumor tissue. The inhibitor was present in the serum IgG fraction. Serum to which excess ESF had been added, after prior neutralization of endogenous ESF with anti-ESF, failed to stimulate erythropoiesis in the polycythemic mouse.

**O**VER 40 % OF ADULT PATIENTS with pure red cell aplasia (PRCA) harbor a thymoma.<sup>1</sup> Earlier attempts to demonstrate a humoral inhibitor of erythropoiesis in the sera or thymic extracts from these patients were unsuccessful.<sup>1-5</sup> More recently, such a factor has been shown by *in vitro* inhibition of cell growth and proliferation in normal marrow,<sup>6</sup> depression of normal erythropoiesis in the mouse<sup>7</sup> and suppression of stem cell colony formation in irradiated mice.<sup>8</sup> In nonthymoma associated PRCA, Krantz and Kao<sup>9</sup> found erythropoietic inhibitory activity in the serum IgG fraction which was lost after the institution of immunosuppressive therapy.

*From the Division of Hematology, Department of Medicine, Montefiore Hospital and Medical Center, Bronx, N.Y.; The Laboratory of Experimental Hematology, Department of Biology, Graduate School of Arts and Science, New York University, New York, N. Y.; Section of Hematology, Morrisania-Montefiore Affiliation, Bronx, N. Y.; and the Department of Medicine (Hematology), Mount Sinai School of Medicine of the City University of New York, New York, N. Y.*

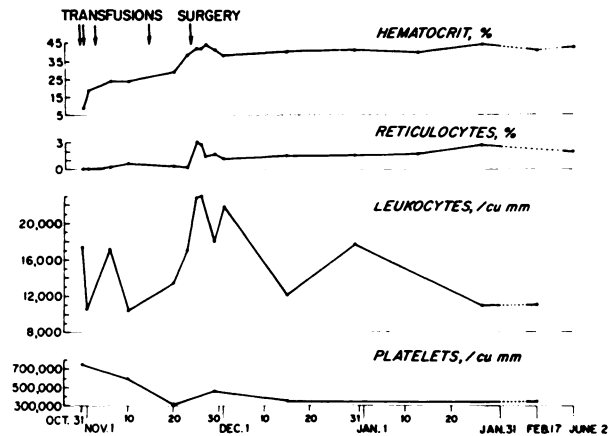
*Submitted January 10, 1971; revised June 9, 1971; accepted June 15, 1971.*

*Supported in part by Grant Ca-5126 from the National Cancer Institute, Grant HE-03357-14 from the National Heart and Lung Institute, USPHS; and the Albert A. List, Frederick Machlin, and Anna Ruth Lowenberg Funds.*

*HAMID AL-MONDHIRY, M.B., Ch. B.: Department of Hematology, Memorial Hospital for Cancer and Allied Diseases, New York, N. Y.; formerly Research Fellow, Division of Hematology, Department of Medicine, Montefiore Hospital and Medical Center, Bronx, N. Y. ESMAIL D. ZANJANI, Ph.D.: Assistant Professor of Medicine, Division of Hematology Mount Sinai School of Medicine, New York, N. Y. MORTON SPIVACK, M.D.: Head of Hematology, Montefiore-Morrisania Affiliation, Bronx, N. Y. RALPH ZALUSKY, M.D.: Associate Professor of Medicine, Mount Sinai School of Medicine, New York, N. Y. ALBERT S. GORDON, Ph.D.: Professor of Biology, Laboratory of Experimental Hematology, Department of Biology, Graduate School of Arts and Science, New York University, New York, N. Y.*

*Address reprint requests to Dr. Zanjani.*

Fig. 1.—Clinical course of the patient before and after removal of the thymoma.



When a thymoma is found in association with PRCA, removal of the tumor has not always led to improvement of the anemia.<sup>10,11</sup> In this report, we describe a patient with the syndrome of PRCA and thymoma whose serum and urine contained an inhibitor of erythropoiesis. Removal of the thymoma was followed by disappearance of this inhibitor and full remission of the anemia. The relationship between this inhibitor and erythropoietin (ESF) was examined.

#### CASE REPORT

A previously healthy 32-yr-old black woman was admitted to Morrisania Hospital on October 31, 1969, with symptoms of severe anemia. There was no history of exposure to toxic drugs and no evidence of bleeding. In March 1968, she underwent subtotal thyroidectomy at another hospital for the removal of a long-standing goiter. Histologically, the thyroid tumor was described as a follicular adenoma. During that hospitalization, she was told that she had a "mass in the chest," but she refused surgery. She was not anemic at that time.

Physical examination was not remarkable except for marked pallor and lethargy. The hematocrit was 9% and no reticulocytes were seen in the peripheral blood. Subsequent blood counts are shown in Fig. 1. Bone marrow aspiration (iliac crest) done on admission and 10 days later showed virtual absence of all erythroid precursors. The megakaryocytes and myeloid series were within normal limits. Lymphocytes, or small lymphocyte-like cells, were noted to be increased diffusely and in clumps. The iron stores were abundant. Thyroid function tests showed the patient to be euthyroid. Lupus erythematosus (LE) cell preparation and antinuclear antibody studies were negative. The direct Coombs' test was positive, 2+, and the indirect was negative. Serum electrophoresis pattern and other laboratory tests were within normal limits. Chest X ray in various projections revealed an anterior mediastinal mass. The patient was transfused with 6 U of packed cells during the few weeks prior to surgery. On November 24th, thoracotomy was performed and a 5 x 7 cm thymic tumor was removed from the anterior mediastinum. Microscopically, the tumor was composed of a mixture of epithelial cells and lymphocytes with the former predominating. Before closing the chest, a biopsy from the lingula of the left lung was taken. Although no gross abnormality was visible in that area, a microscopic focus of metastatic thyroid carcinoma was found.

The patient made an uneventful recovery. One day after the operation the reticulocyte count was 3% and thereafter ranged between 2.8 and 0.7%. Eight days postoperatively, a bone marrow aspirate from the iliac crest revealed many erythroblasts in various stages of development. The myeloid:erythroid ratio was about 3:1. A complete bone survey and chest films showed no evidence of metastatic disease. Two weeks after the operation, the direct Coombs' test was trace positive and it was completely negative 2 wk later. The

patient was followed regularly at the clinic for the next 3 mo. She has been asymptomatic. The hematocrit and reticulocyte count have remained stable at 45% and 1% respectively.

## MATERIALS AND METHODS

### *Preparation of Tumor Extract*

The frozen tumor was homogenized in saline (3 ml/g tissue) in a Waring Blender and the homogenate frozen for 24 hr. The mixture was then thawed and frozen again. This procedure was repeated three times. Following the final thawing, the homogenate was centrifuged at 6000 rpm for 10 min and the sediment rehomogenized in saline (2 ml/g) and frozen. After thawing, the mixture was centrifuged at 7000 rpm for 15 min and the two supernatant fractions combined. The combined mixture was filtered through glass wool and kept frozen until assayed.

### *Assay of ESF*

Erythropoietic stimulating activity of all samples was determined in the exhypoxic polycythemic mouse.<sup>12</sup> Female CF1 mice were rendered polycythemic by exposure to 0.4 atmospheres of air 19 hr/day for a total of 219 hr. Each mouse in the group received a single 1 ml dose of the test material by intraperitoneal injection on day 3 posthypoxia, and 0.5  $\mu$ Ci <sup>59</sup>Fe intravenously on day 5. The per cent RBC-<sup>59</sup>Fe incorporated was determined on day 7.

### *Assay of Erythropoiesis-inhibitory Activity*

*Studies in Normal Mice:* At least five animals per group received either three (urine and serum) or four (tumor extract) daily injections of the test material, with or without pre-treatment with anti-ESF (described below). Whenever possible more animals per group were used. One day after the final injection, each mouse was given 0.5  $\mu$ Ci <sup>59</sup>Fe and the per cent RBC-<sup>59</sup>Fe incorporated was determined 48 hr later.

*Studies in Exhypoxic Polycythemic Mice:* To determine whether the pre- and postoperative sera were capable of blocking the erythropoietic effect of a known quantity of human urinary ESF in the exhypoxic polycythemic mouse, the samples were incubated with a preparation of antihuman urinary ESF (kindly supplied by Dr. J. C. Schooley, University of California, Berkeley, Calif.) to neutralize endogenous ESF. Excess anti-ESF was then removed by precipitation with goat anti-rabbit gamma globulin, (GARCG, purchased from Antibodies, Inc., Davis, Calif.). The amount of GARCG necessary to insure removal of any remaining anti-ESF in these preparations<sup>13</sup> was determined by prior testing against known concentrations of anti-ESF. After ascertaining that the treated samples were devoid of erythropoietic activity, a known amount of exogenous ESF (0.2 IU/ml serum) was added to each sample and the mixture incubated for 2 hr at 37°C.

### *Serum Fractionation*

A 12 ml aliquot of the preoperative serum was subjected to DEAE-Sephadex column chromatography and the IgG separated from the remaining serum proteins.<sup>14</sup> Purity of the IgG fraction was established by immunoelectrophoresis against anti-IgG and quantitated by standard immunodiffusion techniques. Inhibitory activity of both the IgG (Fraction 1) and a concentrate of the remaining serum proteins (Fraction 2) was measured in normal mice.

## RESULTS

Shown in Table 1 are the comparative results of ESF assays in exhypoxic polycythemic mice before and after removal of the thymoma. On admission (preoperative), and prior to transfusion, both the serum and urine possessed considerable ESF activity. Prior incubation of the serum with anti-ESF abol-

**Table 1.—Effect of Serum and Urine on Per Cent RBC-<sup>59</sup>Fe Incorporation in Exhypoxic Polycythemic Mice**

Material Tested	Normal	Patient (Preoperative)	Patient (Postoperative)
Serum (1 ml)	2.40 ± 0.71	26.09 ± 2.32*	9.94 ± 1.78*, † 2.76 ± 0.39 ‡
Urine (2 ml)	2.63 ± 0.93	8.14 ± 1.42°	1.52 ± 0.29
Serum (1 ml), anti-ESF § + 0.2 U ESF	16.70 ± 2.62	4.94 ± 0.99°	9.12 ± 1.35*, † 18.90 ± 2.33 ‡

All values represent mean ± SE for five mice.

\* Significant at  $p < 0.05$ .

† 2 wk postoperative.

‡ 3 mo postoperative.

§ Excess anti-ESF neutralized with GARGG.

ished this activity. In both the immediate postoperative period and 3 mo later ESF activity had decreased toward normal values. Undoubtedly, transfusion of packed red cells shortly after admission (Fig. 1) contributed to this decline. The addition of an excess quantity of ESF, 0.2 IU, to the preoperative serum following neutralization of its endogenous ESF activity with anti-ESF, failed to elicit significant <sup>59</sup>Fe uptake in comparison to normal serum treated in a similar manner.

Erythropoiesis in normal mice was significantly suppressed when animals were injected with the patient's preoperative serum and urine (Table 2). The degree of suppression was essentially unaltered even after prior incubation of the test materials with anti-ESF (excess neutralized with GARGG). These results suggest that the endogenous ESF present in these specimens did not influence the inhibitory activity. Following surgery, the patient's serum no longer showed inhibition of normal mouse erythropoiesis.

Results of the effect of serum protein fractions (preoperative) on normal mouse erythropoiesis are shown in Table 3. Because of the limited quantity of preoperative serum available, the total amount of IgG, Fraction I, administered to each mouse was only half the amount present in the whole serum. This may

**Table 2.—Effect of Serum and Urine on Per Cent RBC-<sup>59</sup>Fe Incorporation in Normal Mice**

Material Tested	Normal	Patient (Preoperative)	Patient (Postoperative)
Serum	21.34 ± 2.16 (16)	7.32 ± 0.97° (6)	21.44 ± 2.91 (7) † 29.26 ± 4.83 (7) ‡
Serum + anti-ESF + GARGG	24.19 ± 2.33 (10)	6.34 ± 1.36° (6)	
Urine	23.18 ± 4.63 (7)	7.13 ± 1.63° (6)	
Urine + anti-ESF + GARGG	19.49 ± 3.47 (10)	4.78 ± 1.10° (12)	

All values represent mean ± SE. Numbers in parentheses represent the number of animals used. Each animal was given either 0.5 ml serum brought to final volume of 1.0 ml with saline or 1.0 ml urine daily for 3 days.

\* Significant at  $p < .01$ .

† 2 wk postoperative.

‡ 3 mo postoperative.

**Table 3.—Effect of Preoperative Whole Serum and Serum Fractions on Per Cent RBC-<sup>59</sup>Fe Incorporation in Normal Mice**

Test Material	RBC- <sup>59</sup> Fe Incorporation
Saline	37.19 ± 2.91
Whole Serum*	5.19 ± 0.67 §
Fraction 1 (IgG)†	18.88 ± 2.39 §
Fraction 2 ‡	27.65 ± 3.67

All values represent mean ± SE for five mice.

\* 0.5 ml serum brought to 1.0 ml with saline given daily for 3 days.

† Final IgG concentration equivalent to that present in 0.25 ml serum (see text).

‡ Composite of remaining serum proteins after removal of IgG.

§ Significant at  $p < .01$ .

account, in part, for the lesser suppression of RBC-<sup>59</sup>Fe uptake in the assay animals given pure IgG as compared with those receiving the whole serum. Fraction 2 consisted of a small amount of residual IgG and the remaining immunoglobulins. The partial inhibitory activity of this fraction may be due to the residual IgG. As shown in Table 4, saline extracts of the thymoma failed to demonstrate any ESF or inhibitory activity.

#### DISCUSSION

Humoral inhibitors of erythropoiesis have now been demonstrated in patients with PRCA both in the presence and absence of a thymoma. Since this form of anemia has developed in some individuals even after the removal of a thymic neoplasm,<sup>10</sup> it has been difficult to attribute an etiologic role to the tumor itself. In this syndrome there is a relatively frequent occurrence of several manifestations of autoimmune disease: positive Coombs' test, antinuclear antibodies, myasthenia gravis<sup>10,15,16</sup> and hypogammaglobulinemia.<sup>17</sup> The erythropoietic inhibitor may represent another autoimmune manifestation.

In many respects the inhibitor is similar to that reported by Jepson and co-workers<sup>7,18</sup> in patients with a thymoma and in nonthymoma patients with PRCA studied by Krantz and Kao.<sup>9</sup> In addition to showing that the inhibitor is an IgG, the latter authors have demonstrated that it is capable of blocking heme synthesis by bone marrow in vitro, and when labeled with fluorescein, shows affinity for normal erythroblastic nuclei. There is no evidence, including our own, that this inhibitor is an antibody to ESF. Although not shown in the data here, we have found that this patient's serum or purified IgG was incapable of replacing known rabbit anti-human ESF in an in vitro hemagglutination

**Table 4.—Per Cent RBC-<sup>59</sup>Fe Incorporation in Normal and Exhypoxic Polycythemic Mice Following Injections of Saline Extracts of Thymoma**

Assay System	Saline Control	Thymoma Extract
Ex-hypoxic polycythemic mice	1.21 ± 0.16	1.32 ± 0.23
Ex-hypoxic polycythemic mice + 0.2 U ESF*	16.70 ± 2.62	14.25 ± 1.02
Normal mice †	21.34 ± 2.16	18.49 ± 3.75

All values represent the mean ± SE of five mice (polycythemic) or eight mice (normal).

\* Single injection of 1 ml of extract preincubated with ESF.

† Extract (1 ml) given daily for 4 days.

system for measuring ESF.<sup>19</sup> A further indication that the inhibitor is unlikely to be an anti-ESF is the observation that both the inhibitor and ESF levels are present simultaneously in the serum.<sup>7,9,19</sup> We have dissociated the effects of these opposing factors on erythropoiesis by utilizing the normal mouse assay for the former and the exhypoxic mouse assay for the latter. In the normal mouse, erythroid precursors in varying stages of development are present, and it would appear that the IgG inhibitor is capable of binding to a specific receptor and interfering with short-term erythropoiesis as measured by <sup>59</sup>Fe incorporation into red cells. In the polycythemic mouse, on the other hand, no recognizable erythroid precursors are present, but ESF-committed stem cells are able to respond to ESF during an interval when inhibitor-sensitive cells are not available. Alternatively, there may be a critical ratio between inhibitor and ESF concentration that determines the relative stimulation or suppression of erythropoiesis in these assay animals. Addition of exogenous ESF, after prior neutralization of endogenous activity, may have been insufficient to overcome this inhibitory activity when specifically examined.

The anemia in this patient was temporally related to the presence of this erythropoietic inhibitor. Since the preoperative specimen was obtained prior to transfusion, this inhibitor is distinctly different from that reported to appear following hypertransfusion.<sup>20</sup> A qualitatively abnormal ESF, ineffective in the patient but active in the polycythemic mouse, is unlikely. Known antibody to ESF abolishes this activity when assayed in the mouse. Furthermore, the release of inhibition of heme synthesis by bone marrow from patients with PRCA that is observed *in vitro*<sup>9</sup> is difficult to interpret on the basis of an abnormal ESF.

#### REFERENCES

- Jacobs, E. M., Hatter, R. V. P., Pool, J. L., and Ley, A. B.: Benign thymoma and selective erythroid aplasia of the bone marrow. *Cancer* 12:47, 1959.
- Ramos, A. J., and Leob, V. J.: Diagnostic problems. *JAMA* 160:1317, 1956.
- Clarkson, B., and Prockop, D. J.: Regenerative anemia associated with benign thymoma. *New Eng. J. Med.* 259:253, 1958.
- Roland, A. S.: The syndrome of benign thymoma and primary regenerative anemia. *Amer. J. Med. Sci.* 274:719, 1964.
- Parry, E. H. O., Kilpatrick, G. S., and Hardisty, R. M.: Red cell aplasia and benign thymoma. *Brit. Med. J.* 1:1154, 1959.
- Barnes, R. D.: Refractory anemia and thymoma. *Lancet* 2:1464, 1966.
- Jepson, J. H., and Lowenstein, L.: Inhibition of erythropoiesis by a factor present in the plasma of patients with erythroblastopenia. *Blood* 27:425, 1966.
- Field, E. O., Caughi, M. N., Blackett, N. M., and Smithers, D. W.: Marrow suppressing factor in the blood of pure red cell aplasia, thymoma and Hodgkin's disease. *Brit. J. Haemat.* 15:101, 1968.
- Krantz, S. B., and Kao, V.: Studies on red cell aplasia. II. Report of a second patient with an antibody to erythroblast nuclei and remission after immunosuppressive therapy. *Blood* 34:1, 1969.
- Schmid, J. R., Kiley, J. M., Harrison, E. G., Jr., Bayrd, E. D., and Pease, G. L.: Thymoma associated with pure red cell aplasia. *Cancer* 18:216, 1965.
- Hirst, E., and Robertson, T. I.: The syndrome of thymoma and erythroblastopenic anemia. *Medicine (Balt.)* 46:225, 1967.
- Camiscoli, J. F., Weintraub, A. H., and Gordon, A. S.: Comparative assay of erythropoietin standards. *Ann. N. Y. Acad. Sci.* 149:40, 1968.
- Schooley, J. C., Zanjani, E. D., and Gordon, A. S.: Immunologic aspects of the biogenesis of erythropoietin. *Blood* 35:276, 1970.
- Sober, H. A., Gutter, F. J., Wyckoff,

M. M., and Peterson, E. A.: Chromatography of proteins. II. Fractionation of serum protein on anion—exchange cellulose. *J. Amer. Chem. Soc.* 78:756, 1956.

15. River, G. L.: Erythroid aplasia following thymectomy. Report of a case with positive lupus erythematosus cell preparation and elevated plasma erythropoietin. *JAMA* 197:726, 1966.

16. Hamilton, C. R., Jr., and Conley, C. L.: Pure red cell aplasia and thymoma. *Johns Hopkins Med. J.* 125:262, 1969.

17. Murray, W. D., and Webb, N. J.: Thymoma associated with hypogammaglobulinemia and pure red cell aplasia. *Amer. J. Med.* 41:974, 1964.

18. Jepson, J. H., Gardner, F. H., Degnan,

T., and Vas, M.: A gammaglobulin inhibitor of erythropoiesis in erythroblastopenic plasma from patients with thymoma. *Clin. Res.* 16:356, 1968.

19. Lange, R. D., McDonald, T. P., Jordan, T. A., Trobaugh, F. E., Jr., Kretchmar, A. L., and Chernoff, A. I.: The hemagglutination-inhibition assay for erythropoietin: A progress report. *In Stohlman, F., Jr. (Ed.): Hemopoietic Cellular Proliferation.* New York, Grune & Stratton, 1970, p. 122.

20. Whitcomb, W. H., and Moore, M. Z.: Physiologic significance of an erythropoietic inhibitory factor appearing in plasma subsequent to hypertransfusion. *Ann. N. Y. Acad. Sci.* 149:462, 1968.