

## BRIEF REPORT: AUTOANTIBODIES AGAINST ERYTHROPOIETIN IN A PATIENT WITH PURE RED-CELL APLASIA

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**A**UTOIMMUNITY is often implicated in pure red-cell aplasia. Approximately 10 to 15 percent of patients with pure red-cell aplasia have thymomas,<sup>1</sup> and remission of the anemia occurs in 25 to 30 percent of these patients after the thymoma is removed.<sup>2</sup> In other patients there are immunologic abnormalities, such as hypogammaglobulinemia,<sup>3</sup> monoclonal immunoglobulins,<sup>4</sup> antithyroid antibodies,<sup>5</sup> antinuclear antibodies,<sup>6,7</sup> and autoimmune hemolytic anemia.<sup>3,8</sup> Immunosuppressive therapy is successful in many patients,<sup>2,9-12</sup> and a good response to plasmapheresis has been reported in two patients.<sup>13,14</sup>

In 1967 Krantz and Kao reported that plasma from a patient with pure red-cell aplasia inhibited heme synthesis by normal bone marrow cells *in vitro*.<sup>7</sup> Further studies<sup>4,15,16</sup> demonstrated an IgG antibody against erythroblasts in patients with the disease. More recently, Messner et al.<sup>14</sup> described a patient with a plasma factor that blocked the differentiation of erythroblastic colonies. T cells also appear to be important in the disease,<sup>17,18</sup> and a role for suppressor T cells has been suspected in the form of pure red-cell aplasia that occurs in association with chronic lymphocytic leukemia.<sup>19-21</sup>

Two patients who may have had a serum inhibitor against erythropoietin have been described.<sup>22,23</sup> However, since these studies were undertaken with unpurified erythropoietin it is difficult to be sure of the specificity of the antibody. We describe a patient with pure red-cell aplasia with antierythropoietin antibodies. These antibodies inhibited the binding of erythropoietin to its receptor and blocked the differentiation of erythroid progenitors *in vitro*.

### CASE REPORT

A 70-year-old woman was admitted to the hospital because of severe anemia. The medical history was notable only for hypertension. There was no history of fever, rash, or exposure to drugs or toxic agents thought to interact with erythrocytes or erythroblasts. The clinical examination was normal, with no organomegaly noted.

The hemoglobin level was 5.7 g per deciliter, with a red-cell count of 1.9 million per cubic millimeter. The mean corpuscular volume was 83.4  $\mu\text{m}^3$ , with a mean corpuscular hemoglobin concentration of 28.8 g per deciliter. No reticulocytes were found on several examinations. White-cell and platelet counts were normal. The bone marrow showed pure red-cell aplasia. There were no signs of dysmyelopoiesis

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or abnormal lymphoid proliferation. Bone marrow cellularity was normal. The granulocytes and megakaryocytes appeared to be normal. Thoracic and abdominal computed tomographic scans showed no evidence of thymoma, lymphoma, or solid tumor.

Serologic tests for the human immunodeficiency virus, cytomegalovirus, hepatitis, and Epstein-Barr virus were negative. Tests for the human B19 parvovirus involving serologic and dot blot analyses and amplification of viral DNA with the polymerase chain reaction were negative.

A direct Coombs' test and a test for antinuclear antibodies were negative. The results of electrophoresis of hemoglobin were normal. Vitamin B<sub>12</sub>, folate, and creatinine concentrations were normal.

Symptomatic therapy began with the administration of phenotyped, filtered red-cell concentrates. The transfusion requirements were about 4 units of packed red cells per month. The patient declined to receive immunosuppressive therapy.

Nine months after the initial presentation, the reticulocyte count spontaneously increased. The transfusion requirement diminished and was ultimately eliminated after 18 months of observation. As of this writing, the patient is hematologically normal.

### METHODS

#### Bone Marrow Cultures

Informed consent was obtained, and the patient's bone marrow was collected in sterile vials containing 200 U of preservative-free heparin. Cells with a density of 1.077 g per cubic centimeter were isolated by Ficoll centrifugation. Erythroid cultures were established according to the plasma-clot culture technique<sup>24</sup> with some modifications. The cells were plated at a final concentration of  $1 \times 10^5$  per milliliter in petri dishes (30 by 10 mm) containing alpha medium (Flow Laboratories, ICM, Irvine, Scotland), 1 percent deionized bovine serum albumin (Sigma, St. Louis) prepared according to the methods of McLeod et al.,<sup>24</sup> 10 percent L-asparagine (0.2 mg per milliliter; Calbiochem, La Jolla, Calif.) diluted in alpha medium plus calcium chloride (28 mg per deciliter), and 10 percent bovine citrated plasma (GIBCO, Paisley, Scotland). Agar-leukocyte-conditioned medium (10 percent) and various concentrations of recombinant human erythropoietin

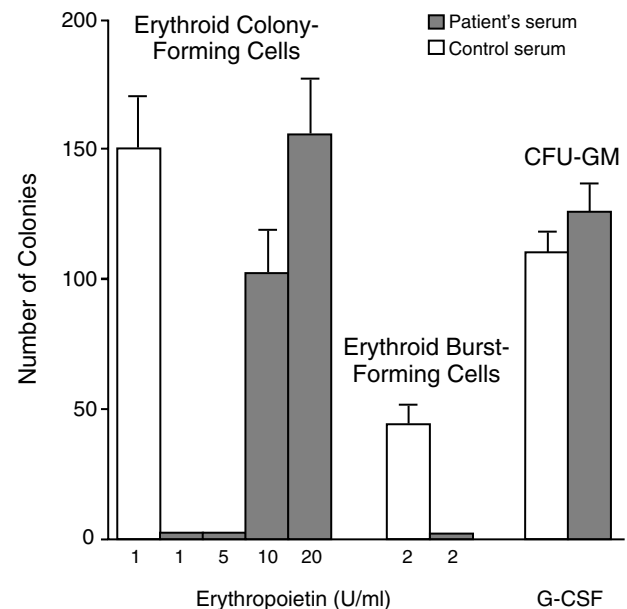


Figure 1. Effect of the Patient's Serum on the Growth of Autologous Hematopoietic Progenitors.

*In vitro* studies were performed at diagnosis. The values are the mean ( $\pm$ SD) numbers of colonies formed. Erythroid progenitors and granulocyte-macrophage colony-forming units (CFU-GM) were cultured with either control serum or the patient's serum at a final concentration of 20 percent in the presence of different concentrations of erythropoietin (1 to 20 U per milliliter) or 200 ng of granulocyte colony-stimulating factor (G-CSF) per milliliter.

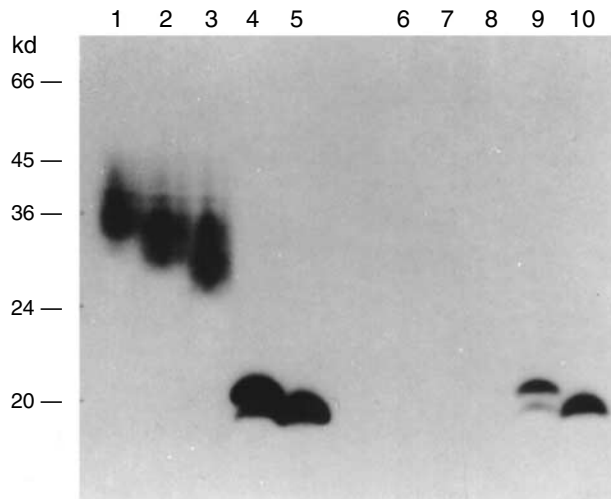


Figure 2. Immunoprecipitation of Native or Deglycosylated  $^{125}\text{I}$ -Labeled Erythropoietin by the Patient's Serum.

Native  $^{125}\text{I}$ -labeled erythropoietin (lanes 1 and 6) was sequentially deglycosylated with a sialidase alone (lanes 2 and 7), a sialidase and an *O*-glycosidase (lanes 3 and 8), a mixture of endoglycosidase F and *N*-glycosidase F (lanes 4 and 9), or the three glycosidases together (lanes 5 and 10). Both native and deglycosylated erythropoietin were incubated overnight with the patient's serum (lanes 1 through 5) or control serum (lanes 6 through 10). Then, antibody-bound erythropoietin was recovered with formalin-fixed *S. aureus* and analyzed by polyacrylamide-gel electrophoresis and autoradiography. The migration positions of unlabeled molecular-mass markers are indicated on the left-hand side of the figure.

(Cilag, Paris) were added to the cultures. The clots were fixed at day 7 or day 14 of culture and stained with benzidine and hematoxylin. Granulocytic colonies were grown in methylcellulose medium (0.8 percent methylcellulose in Iscove's medium; Terry Fox Laboratories, Vancouver, B.C., Canada) with 1 percent deionized bovine serum albumin. Agar-leukocyte-conditioned medium at a final concentration of 10 percent and recombinant granulocyte colony-stimulating factor (200 ng per milliliter; Amgen, Thousand Oaks, Calif.) were added to the cultures. Granulocytic colonies were assessed on day 14. Erythroid and granulocytic colonies were grown either with normal pooled serum from 10 healthy volunteers as a control or with the patient's serum at a final concentration of 20 percent. All the cultures were incubated in a fully humidified atmosphere supplemented with 5 percent carbon dioxide. After 7 days of incubation for erythroid colony-forming cells or 14 days for erythroid burst-forming cells, the clots were fixed and stained with benzidine and hematoxylin.

#### Measurement of Serum Erythropoietin

Serum erythropoietin was measured by enzyme-linked immunosorbent assay with a commercial kit (Bio-Merieux, Marcy l'Etoile, France).

#### Binding of $^{125}\text{I}$ -Labeled Erythropoietin

Highly purified recombinant human erythropoietin, iodinated as previously described,<sup>25</sup> had specific activities ranging from  $2.5 \times 10^7$  to  $5 \times 10^7$  cpm per microgram. Different con-

centrations of  $^{125}\text{I}$ -labeled erythropoietin were incubated overnight at  $4^\circ\text{C}$  with  $10 \mu\text{l}$  of the patient's serum or control serum in a total volume of  $200 \mu\text{l}$  of phosphate-buffered saline containing 0.1 percent Triton X-100. Saturating amounts of formalin-fixed *Staphylococcus aureus* were added, and the tubes were incubated for another 20 minutes while being stirred continuously. Then, 2 ml of ice-cold phosphate-buffered saline containing 0.1 percent Triton X-100 was added, and the tubes were centrifuged for 15 minutes at  $1500 \times g$ . The resulting pellet was washed twice, and the radioactivity was counted. The same protocol was used for Scatchard analysis, except that at the end of the incubation, the mixture was centrifuged without phosphate-buffered saline and an aliquot of the supernatant was counted to determine free radioactivity.

#### Deglycosylation Studies

Erythropoietin was deglycosylated as previously described.<sup>26</sup> In brief,  $^{125}\text{I}$ -labeled erythropoietin ( $0.25 \mu\text{g}$ ) was diluted with  $200 \mu\text{l}$  of 50 mM sodium phosphate buffer (pH 5.0) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM *o*-phenanthroline, 0.1 percent Triton X-100, and 0.02 percent sodium azide. Then, 50 mU of *Arthrobacter ureafaciens* neuraminidase was added, and the mixture was incubated for one hour at  $37^\circ\text{C}$ . Next, 7.5 mU of *O*-glycosidase and 500 mU of a mixture of endoglycosidase F and *N*-glycosidase F (Boehringer-Mannheim, Mannheim, Germany) were added, and the incubation was continued for 18 hours at  $37^\circ\text{C}$ . The reaction mixtures then underwent chromatography on Sephadex G25 columns equilibrated with phosphate-buffered saline containing 0.02 percent Tween 20.

#### RESULTS

The patient's serum erythropoietin level, measured on several occasions at the time of diagnosis, was low for this degree of anemia (5 to 10 mU per milliliter; range in our laboratory in patients without anemia, 5 to 25 mU per milliliter), and the hemoglobin level was be-

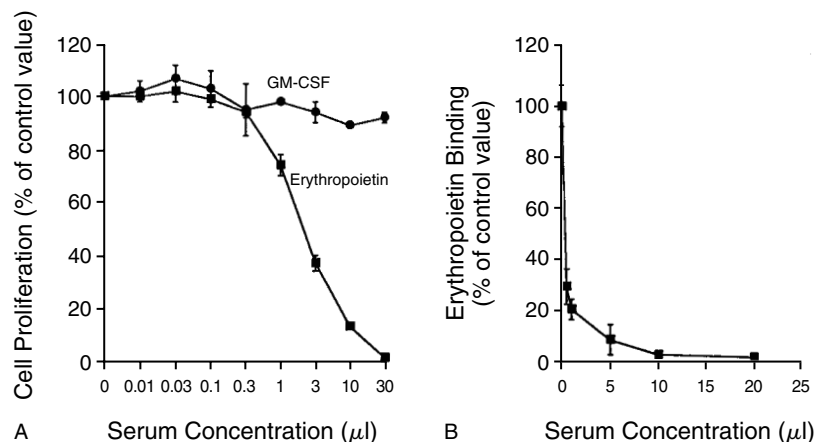


Figure 3. Inhibition of Erythropoietin-Induced Cell Proliferation and  $^{125}\text{I}$ -Labeled Erythropoietin Binding to UT-7 Cells by the Patient's Serum.

In Panel A, 5000 UT-7 cells were seeded in  $120 \mu\text{l}$  of culture medium containing either 2.5 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF) per milliliter or 0.5 U of erythropoietin per milliliter and the indicated amounts of the patient's serum (x axis). The cells were cultured for three days, and cell proliferation was determined with a fluorescent dye (Alamar blue, Interchim, Montluçon, France). The results are expressed as a percentage of control values in cells cultured without the patient's serum and represent the mean ( $\pm$ SD) values of three determinations. In Panel B, the patient's serum was diluted with normal serum and  $20 \mu\text{l}$  of the mixture containing the indicated amounts of the patient's serum (x axis) was incubated with 40,000 cpm of  $^{125}\text{I}$ -labeled erythropoietin for 15 minutes at  $37^\circ\text{C}$ . Then, 1 million UT-7 cells were added, and the incubation was continued for another 30 minutes at  $37^\circ\text{C}$ . The cells were washed with ice-cold phosphate-buffered saline, and cell-bound radioactivity was measured. Specific binding was determined by subtracting the amount of nonspecific binding measured with the use of a 100-fold molar excess of unlabeled erythropoietin. Each point represents the mean ( $\pm$ SD) of three determinations. The control value of 100 percent specific binding is  $6630 \pm 590$  cpm.

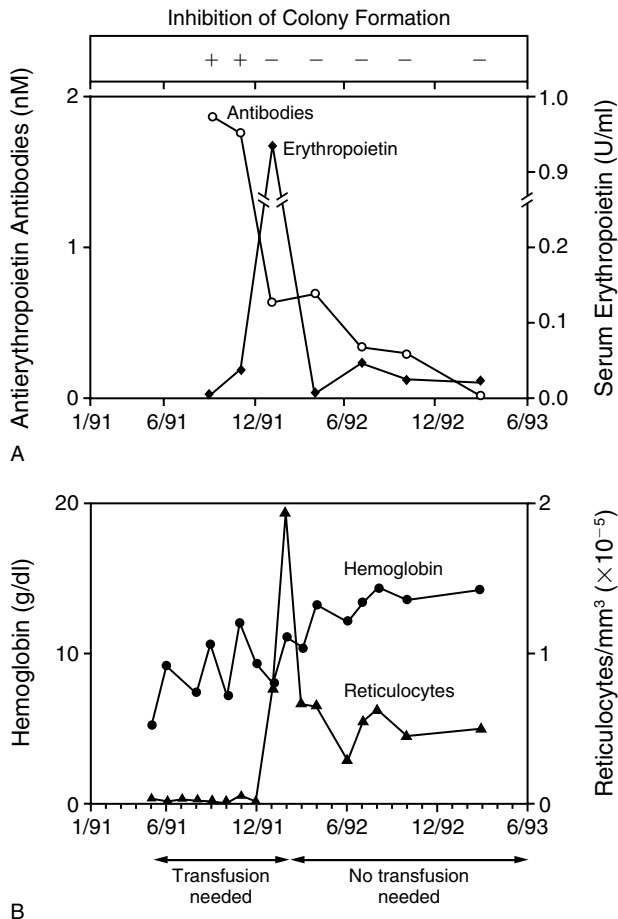


Figure 4. Changes in Hematologic Variables during Follow-up of a Woman with Pure Red-Cell Aplasia.

Panel A shows the changes in the antierythropoietin antibody titer, the serum erythropoietin level, and the status of inhibition of erythroid-colony formation in standard culture conditions (erythropoietin concentration, 1 IU per milliliter). A plus sign denotes the presence of inhibition, and a minus sign its absence. Panel B shows the changes in the hemoglobin concentration, reticulocyte count, and transfusion requirements.

tween 5.2 and 6.1 g per deciliter. This finding was surprising because serum erythropoietin levels are usually very high in pure red-cell aplasia.<sup>1</sup>

Cultures of the patient's bone marrow cells in normal serum yielded normal numbers of erythroid and granulocytic progenitors (erythroid colony-forming cells, erythroid burst-forming cells, and granulocyte-macrophage colony-forming units) (Fig. 1). In contrast, when the patient's bone marrow cells were cultured in autologous serum, the growth of erythroid progenitors was completely inhibited, whereas there was no inhibition of granulocytic progenitors (Fig. 1). Erythropoietin (1 to 20 U per milliliter) in the culture medium reversed the inhibition of the growth of erythroid progenitors by autologous serum (Fig. 1). The same results were obtained when normal bone marrow cells were cultured in the presence of the patient's serum (data not shown).

The results with bone marrow cultures and the serum erythropoietin level suggested the presence of an antibody that was capable of neutralizing erythropoietin. We therefore sought antierythropoietin antibodies in the

patient's serum. The patient's serum bound <sup>125</sup>I-labeled erythropoietin in the presence of formalin-fixed *S. aureus*, indicating the presence of IgG antierythropoietin antibodies. In contrast, several hundred control serum samples had only background levels of bound <sup>125</sup>I-labeled erythropoietin. The antierythropoietin antibodies in serum collected at diagnosis were typed with an enzyme-linked immunosorbent assay. The patient's serum or control serum was incubated with erythropoietin-coated microplates, and antierythropoietin antibodies were revealed by class-specific peroxidase-labeled second antibodies (Difco Laboratories, Detroit). With this method, only IgG antierythropoietin antibodies were found in the patient's serum (data not shown). Scatchard analysis of the affinity of the antibodies revealed an apparent single class of <sup>125</sup>I-labeled erythropoietin-binding sites with a dissociation constant at equilibrium of  $430 \pm 80$  pM (the mean  $[\pm SD]$  of three independent determinations) and a maximal concentration of binding sites of  $1.52 \pm 0.25$  pmol per milliliter of serum (data not shown).

Figure 2 shows that the antibodies bound to fully deglycosylated erythropoietin and thus were directed against the protein moiety of erythropoietin. Precipitation of small amounts of *N*-deglycosylated erythropoietin in control serum (lanes 9 and 10 of Fig. 2) was also observed in the absence of serum (data not shown) and reflects the tendency of *N*-deglycosylated erythropoietin to aggregate and precipitate.<sup>27</sup>

The patient's serum was tested with UT-7 cells, a human cell line that responds to erythropoietin<sup>28</sup> and expresses a large number of erythropoietin receptors.<sup>29</sup> Figure 3 shows that the patient's serum completely inhibited the binding of <sup>125</sup>I-labeled erythropoietin to UT-7 cells and the erythropoietin-induced proliferation of UT-7 cells.

In vitro studies of normal marrow cultured with the patient's serum, measurements of the serum erythropoietin level, and quantitation of antierythropoietin antibodies were performed during the evolution of the illness. In vitro inhibition of the growth of erythroid colonies was observed both when the antierythropoietin antibody titer was high and when the serum erythropoietin level was low, at a time when the patient was receiving 4 units of red-cell concentrates per month. Later, the antierythropoietin antibody titer decreased while the serum erythropoietin concentration simultaneously increased; in vitro inhibition of the growth of erythroid colonies was not found. The transfusion requirement decreased, and the reticulocyte count increased sharply (Fig. 4). Ultimately, antierythropoietin antibodies became undetectable, and the serum erythropoietin concentration, reticulocyte count, and hemoglobin level stabilized at normal values. Transfusions were no longer required.

## DISCUSSION

We report finding antierythropoietin antibodies in a patient with pure red-cell aplasia. The presence of antibodies against the protein backbone of the erythropoietin molecule in the patient's serum was ascertained by biologic and biochemical methods. The patient's se-

rum inhibited the growth of erythroid colonies without inhibiting the growth of progenitors of other lineages, and this inhibition was completely reversed by high concentrations of erythropoietin. Moreover, the patient's serum contained an IgG antibody that bound to both native and deglycosylated erythropoietin, inhibited the binding of erythropoietin to the erythropoietin receptor, and blocked the ability of erythropoietin to induce the growth of an erythropoietin-responsive cell line. The concentration of antierythropoietin antibodies in the patient's serum was relatively low, but corresponded to a binding capacity of 2.7 U of erythropoietin per milliliter of serum; the measured equilibrium dissociation constant of the serum antibodies was very low — close to that of the erythropoietin receptor itself.<sup>26</sup> This low equilibrium constant strongly suggests that the antibodies were able to neutralize most of the circulating erythropoietin molecules. Indeed, the erythropoietin level in the patient's serum was very low, an unusual finding in pure red-cell aplasia.

Erythroid progenitors, erythroblasts, and erythropoietin are each potential targets of inhibitors of erythropoiesis in acquired pure red-cell aplasia. The high levels of erythropoietin that are usually present in the plasma of patients with the disease indicate that the inhibition is most likely directed at erythroid cells in the marrow, and several studies have reported the presence of IgG antibodies against erythroblasts or erythropoietin-responsive cells.<sup>4,15,16</sup> Two unusual cases of pure red-cell aplasia and low serum erythropoietin levels have been described. In 1968, Jepson and Lowenstein<sup>22</sup> raised the possibility of an antierythropoietin inhibitor in one case of pure red-cell aplasia, but the means of testing their idea were not available. In 1975 Peschle et al.<sup>23</sup> described a patient they suspected of having antierythropoietin antibodies. The administration of this patient's IgG to mice induced severe anemia without increasing erythropoietin levels. Before therapy, no erythropoietin activity was detectable in the patient's serum. After acidification and boiling of the serum to denature the IgG, the erythropoietin activity increased greatly. However, since purified erythropoietin was not available, a direct demonstration of antierythropoietin antibodies was not possible.

The course of the pure red-cell aplasia in our patient mimics the evolution of transient erythroblastopenia in children. That disorder is often associated with autoantibodies against erythroid progenitors.<sup>30</sup> Transient erythroblastopenia in children may be associated with viral infection. It is possible that the transient appearance of an antierythropoietin antibody in our patient was related to such a mechanism.

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