Decreased *in Vivo* and *in Vitro* Erythropoiesis Induced by Plasma of Ten Patients with Thymoma, Lymphosarcoma, or Idiopathic Erythroblastopenia

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SUMMARY

Plasma was collected from 10 patients with severe anemias of a variety of etiologies, and their effect on *in vivo* erythropoiesis and nucleoprotein and heme synthesis in human bone marrow cultures was studied. The pathological factor in most cases appeared to be immunoglobulin G. Demonstration of the effect of this inhibitor was often dependent upon dilution of plasma to reduce the concentration of erythropoietin or upon its separation from the erythropoietin content of the plasma sample, since the presence of the latter in high concentrations tended to mask the effect of the inhibitor. Plasma (and extracts thereof) collected from patients with lymphomatous disease and thymoma appeared to be a more potent inhibitor of erythropoiesis. An extract of thymoma also displayed potent erythropoietic inhibitory effect. The results appeared to indicate that the effective immunoglobulin damaged erythroblasts much as serum antibodies damage circulatory erythrocytes in autoimmune hemolytic anemia, leading to the clinical picture of erythroblastopenia.

INTRODUCTION

The occurrence of erythroblastopenia, first described in association with thymoma in 1928 (28), has long been a medical curiosity. Since that time, the number of clinical cases reported in the literature have been few, but they continue to intrigue the clinician primarily because of the association of this anemia with that immunological organ, the thymus. The clinical aspects of this syndrome have recently been reviewed by Hirst and Robertson (13), and some of the experimental data and factors related to the induction of the disease have been studied by others (8, 15, 17). The means by which the clinical state is induced remains totally obscure. However, it was postulated, on the basis of previous speculations and the association with thymoma, that an autoimmune mechanism was involved (8). In 1966 a factor that inhibited the induction of erythropoiesis (20) was found in the plasma of 2 patients with erythroblastopenia, one of whom had a thymoma. In the same year (5), inhibition of bone marrow proliferation by the plasma of a patient with the erythroblastopenia-thymoma syndrome was reported at the meeting of the British Society for Hematology; this has been subsequently discussed elsewhere (3, 4). However, while there was much discussion as to the nature and possible mechanism of action of such factors in the production of the clinical syndrome, there were few data precisely defining the problem. Up to that time, the data of Barnes (3, 5) and of others (8, 20) associated with the clinical picture of hyperglobulinemia, eosinophilia, and plasma cell and lymphocyte infiltration of the bone marrow only strongly suggested that this could be an autoimmune process. The previously observed clinical response of some patients to corticoids (13), with relapse upon their discontinuation (12), remission of erythroblastopenia associated with tumors following radiotherapy (9, 10) and surgical excision (2, 13), certainly suggests a more than casual relationship between the tumor and erythroblastopenia. Other cases of erythroblastopenia associated with a plasma inhibitor have been reported with carcinoma of the bronchus (9) and Hodgkin’s disease (10). Subsequently, a series of patients with erythroblastopenia, with and without thymoma, who have had inhibitors to erythropoiesis in their plasma have been reported (2, 14, 18, 19, 21, 22–25, 29, 37), and some of these patients went into remission following the use of immunosuppressants (6, 24, 25, 29). Krantz and Kao (24) more clearly defined, at least in part, the nature of the factor in the plasma when they showed that an IgG fraction from plasma of a patient with idiopathic erythroblastopenia inhibited heme synthesis in human bone marrow cultures and caused immunofluorescence of erythroblast nuclei. These data, in conjunction with those reported earlier by Barnes (3) and Barnes and Xefteris (5), provided more definitive evidence that the factor acted directly on bone marrow cells and was an immunoglobulin. Subsequently, these authors published several case reports with the same collaborative *in vitro* results (22, 23, 25, 29, 37). Both *in vitro* and *in vivo* data have also been reported that indicate that the plasma of patients with thymoma contains an IgG that inhibits erythropoiesis and DNA synthesis (18, 21).
In view of the small number of cases published in the literature, the authors through the cooperation of several clinicians collected material from 10 patients with clinically proven erythroblastopenia and 2 patients with anemia of bone marrow failure. Four of the patients had associated thymoma or lymphosarcoma, 6 had erythroblastopenia of unknown etiology, 1 had sideroblastic anemia which later terminated in leukemia, and 1 had drug-induced hypoplastic anemia. The object of the project was to determine how consistently one could demonstrate the presence of inhibitors to erythropoiesis in the plasma of these patients, whether the inhibitor was an immunoglobulin, what technical factors influenced their detection in various assay systems, and what optimum conditions were required to demonstrate these factors. Consequently, the erythropoietic inhibitory effect of whole and fractionated plasma samples collected from patients with erythroblastopenia was studied by measuring their effect on the induction of erythropoiesis in vivo and on nucleoprotein, protein, and heme synthesis in human bone marrow cultures. This was concomitantly compared with normal plasma similarly treated (21).

**MATERIALS AND METHODS**

Plasma samples and, when available, thymoma tissue were frozen and shipped in Dry Ice by the many physicians who were kind enough to supply material for these investigations. All samples were maintained in the frozen state until processed. The protein content and immunoglobulin concentration of test materials were determined by standard procedures. The erythropoietin content of whole plasma and of fractions thereof was determined in the polycythemic mouse assay as described previously (16). Plasma was lyophilized and fractionated on a Sephadex G-100 column in 0.1 M ammonium bicarbonate buffer at pH 8. γ-Globulin fractions of whole plasma (IgG) were obtained by elution from DEAE-cellulose (33). One-half of the sample was lyophilized and the other one-half was concentrated in Visking casing (m.w. pore size, 20,000) overnight against 10 volumes of Medium 199. The fractions were subjected to electrophoresis on cellulose acetate, immunoglobulin was subjected to analysis by immunodiffusion in gel against appropriate antisera, and their protein content was determined by standard procedures. Normal plasma was similarly fractionated, tested, and used in all experiments as controls. These normal plasma fractions exhibited no inhibitory or stimulatory effect in any of the systems used. All samples were passed through a 0.45-μm Millipore filter prior to their use.

In those experiments performed to determine the possible inhibitory effect of plasma fractions on erythropoiesis induced by injection of erythropoietin, the procedure was as follows. Mice were given injections of 1 to 2 mg of Imferon i.p., then were exposed for 3 weeks to 345 mm mercury atmospheric pressure, and were removed on Day 0. On the 4th day posthypoxia, either 2 mg of the IgG in PBS or 5 mg of the void volume of the Sephadex column were injected i.p. concurrently with a s.c. injection of a standard dose of erythropoietin, followed by 2 mg of the test material (IgG) on the following morning and again 24 hr later. 59FeCl3, 0.5 μCi in 0.1 ml PBS, was injected via the tail vein 56 hr after injection of the erythropoietin. The percentage incorporation of radioiron into erythrocytes at 48 hr was determined as a measure of erythropoietic activity.

Thymoma tissue was homogenized in PBS, kept at a neutral pH of 7 and at 4° during processing. The material was then spun at 3000 rpm for 10 min in the cold, the supernatant was harvested, and the pH was adjusted to pH 7. The sediment was washed twice more with cold PBS and respun, and the supernatant was added to that previously collected. It was then dialyzed in Visking casing (m.w. pore size, 20,000) overnight against 10 volumes of Medium 199 in the cold, and then was concentrated by ultrafiltration. Prior to use, the material was passed through an 0.45-μm Millipore filter. The protein concentration was rechecked and was in close agreement with that prior to filtration. The protein concentration, immunoglobulin content, and electrophoretic pattern of the final material were determined. A fraction of the material was tested for antierythropoietin activity by immunodiffusion in gel against erythropoietin.

The γ-globulin and thymoma extracts were tested for biologically active anterythropoietin activity as follows. The patient's separated erythropoietin, fractionated on Sephadex G-100 and previously tested for erythropoietin content in polycythemic mice, was incubated with the purified IgG by the method described by Schooley and Garcia (32). The supernatant was tested for its erythropoietin content. The patient's erythropoietin was simultaneously incubated with normal γ-globulin in a similar manner and served as a control.

**In Vitro Experiments**

Bone marrow was obtained from patients with no hematological abnormalities and from patients with various anemias. Leukemic marrow was not studied. A standard procedure is described. In those experiments in which an adjusted concentration of plasma or extracted γ-globulin fractions were added, the volumes were adjusted accordingly.

1. **Heme Synthesis**

This method, from our laboratory, was previously described (35).

A. **Cell Suspension.** Bone marrow was aspirated from the posterior iliac crest and was placed immediately in a centrifuge tube containing 10 ml of Hanks' balanced salt solution (Microbiological Associates, Bethesda, Md.) and a few drops of heparin. After centrifugation of the marrow specimen at 500 × g at room temperature for 10 min, the supernatant was discarded and the cells were resuspended in 3 ml of Hanks' solution. The nucleated cells were counted (standard white blood cell counting technique), and their
number was adjusted to 8 to 10 \times 10^{10}/\text{ml} with Hanks' solution.

B. \textit{\textsuperscript{55}Fe-labeled Serum}. \textit{\textsuperscript{55}FeCl}_3 (Abbott Laboratories Ltd., Montreal, Canada) was diluted with 0.9% NaCl solution so that 1 ml contained 0.1 to 0.2 \mu g Fe, with a range of specific activity of 4.4 to 11.0 \mu Ci/\mu g. One ml of this solution was added to 4 ml of serum (normal serum of the same ABO group as the bone marrow), and the mixture was allowed to equilibrate for 30 min at room temperature.

Disposable 15- x 175-mm plastic tubes were used as culture vessels. All procedures were performed under aseptic conditions.

To set up the cultures, 0.6 ml of cell suspension, 0.4 ml of the plasma to be tested, and finally 1.0 ml of \textit{\textsuperscript{55}Fe-labeled serum} were added to each tube to give a final volume of 2.0 ml. Control tubes containing normal plasma from the same bone marrow were prepared under identical conditions.

Cell counts were performed in duplicate, and smears were made from another tube that was prepared similarly but to which no radioactive iron was added. The nucleated cells counts varied from 2 to 4 \times 10^{10}/\text{ml} of culture fluid. Smears were stained with Jenner-Giemsa. The percentage of erythroblasts in each smear was determined by the differential count of 1000 nucleated cells. ("Erythroblasts," as used in this paper, include all nucleated red cells.) The calculated absolute number of erythroblasts in the cultures ranged from 2 to 8 \times 10^9/ml.

The cultures were incubated at 37°C and were rotated at 12 rpm for 24 hr. At the end of the incubation period, the cells were washed 3 times with chilled 0.9% NaCl solution and were hemolyzed with 2 ml of distilled water at 4°C. Hemolysis was always complete after 30 min. The radioactivity of the culture then was measured in a well-type scintillation counter (Atomic Instrument Co., Cambridge, Mass.). The standard was prepared from the \textit{\textsuperscript{55}Fe-labeled serum} by dilution to 1:20 with distilled water.

Initially, a count was performed upon the whole hemolysate, containing both supernatant and stroma. From this count and from the known concentration of iron in the culture, the total iron uptake by the cells was calculated and expressed in \mu g iron per erythroblast per hr. This value represents all of the iron removed from the medium by the cells; it includes the iron bound to the cell membrane and the intracellular nonhemoglobin iron as well as the hemoglobin iron. Uptake by cells other than erythroblasts was comparatively insignificant and therefore was not taken into consideration in the calculations.

The tubes then were centrifuged at 3000 \times g for 15 min, and the supernatant was assayed for radioactivity to determine the amount of iron incorporated into heme; this was expressed in \mu g iron per erythroblast per hr. This value may be considered to represent the heme iron, since only about 10% of the supernatant iron is bound to microsomes and mitochondria (1).

II. Incorporation of Tritiated Thymidine, Uridine, and Leucine

A. Cell Suspension. Bone marrow from the posterior iliac crest was aspirated into a heparinized syringe and immediately transferred into a disposable sterile plastic tube (Falcon Plastics, Oxnard, Calif.). The tube was placed in an incubator at 37°C for about 30 min. After the red cells sedimented, the supernatant plasma, containing nucleated cells, was transferred into a new tube. Cell counts were performed and were adjusted to 2 to 3 \times 10^{9} cells/ml with Hanks' balanced salt solution (Microbiological Associates) containing 20 to 30% autologous plasma.

B. Isotope Solution. Thymidine\textsuperscript{4}H (8.3 \mu Ci/mg), uridine\textsuperscript{4}H (102 \mu Ci/mg), and leucine\textsuperscript{3}H (38.4 \mu Ci/mg) (New England Nuclear, Boston, Mass.) were diluted with 0.9% NaCl solution to contain 40 \mu Ci/ml.

For preparation of the cultures, 1.5 ml cell suspension, 0.4 ml of plasma to be tested, and 0.1 ml of radioactive solution were added to the tubes to a final volume of 2 ml. In some experiments, these volumes were doubled to a final volume of 4 ml. Tubes to which normal plasma was added, but which were prepared from the same marrow, served as controls. The cultures were incubated at 37°C and rotated in a roller drum at 12 rpm for 24 hr. Duplicate, triplicate, or quadruplicate tubes were prepared in every experiment. The isotopes were added for 2 hr at the end of the incubation period.

Cellular nucleic acids and protein were extracted and measured by a modification of the methods of Schmidt and Thannhauser (30), Schneider (31) and Lowry et al. (26) as follows. The cells were washed 3 times with chilled 0.9% NaCl solution; then an equal volume of 10% TCA was added, and the precipitate was washed twice with 5% TCA. The supernatants were discarded, and precipitate containing RNA, DNA, and protein was treated with 0.5 ml of 0.5 \textit{N} KOH at 37°C overnight. Then an equal volume of 10% TCA and one-tenth the volume of 6 \textit{N} HCl was added, at room temperature. After centrifugation, the supernatant was pooled with the supernatants of the 2 consecutive washings with 5% TCA. This fraction contained the RNA. To the residue, 1.5 ml of 5% TCA were added and heated to 90°C for 20 min, then were centrifuged, and the supernatant and 2 consecutive 5% TCA washings were collected. This fraction contained the DNA. The TCA was removed from the RNA and DNA fractions by vigorous shaking (Vortex mixer; Fisher Scientific Co., Pittsburgh, Pa.) with 10 volumes of ether. The final residue containing the protein was dissolved in 1 ml of 0.2 \textit{N} ammonium hydroxide.

Each fraction was then divided into 2 aliquots. One aliquot was used for measuring the concentrations of nucleic acid and protein. The concentration of nucleic acid was determined by measuring absorbance in a Beckman spectrophotometer at 260 nm. Protein concentration was determined by the method of Lowry et al. (26). To the other aliquots, 15 ml of dioxane-based scintillation solution (7) (100 g naphthalene, 7 g PPO, and 300 mg POPOP in 1000 ml 1-4 Dioxan) were added and the radioactivity was determined in a liquid scintillation counter. Results were expressed as cpm/\mu g DNA, RNA, or protein. Counting efficiency was determined by the external standard method and was the same in the controls as in the experimental cultures.
RESULTS

I. Characterization of Materials Used in the in Vivo and in Vitro Experiments

A. Erythropoietin Concentration in Plasma and Characteristics of the Extracted Fractions. Normal plasma contained no significantly detectable concentration of erythropoietin by the method used. Plasma collected from patients with erythroblastopenia contained increased concentrations ranging from a low of 0.1 unit/ml to a high of more than 3.3 units/ml (Tables 1 and 2). Three of the 4 patients with thymoma or lymphosarcoma had much lower concentrations of erythropoietin in their plasma than expected (Table 2). The erythropoietin could be effectively separated from the larger-molecular-weight immunoglobulins by molecular sieve chromatography (Chart 1). The immunoglobulins were excluded from the column and found in the void volume ($V_0$). They were characterized by immunodiffusion in gel as IgM, IgG, and IgA. No erythropoietic activity was found in these fractions when assayed in the polycythemic mouse assay. The erythropoietic activity was limited to fractions 32 to 43, with the peak activity occurring in the molecular weight range of 59,000 on the descending limb of the albumin peak. This is in agreement with that reported previously (27). More units were eluted from the column that were calculated from the erythropoietic effect of the whole plasma applied to the column that had been assayed in polycythemic mice. This indicated that some type of inhibitory material was removed from the unpurified whole plasma. The fraction containing erythropoietin obtained by this method was run in polyacrylamide gel on preparative disc electrophoresis. This was an extremely heterogeneous material with at least 2 separate bands that contained erythropoietically activated material (unpublished data).

More conveniently, immunoglobulin was obtained by elution from DEAE-cellulose which was found to be pure IgG by immunodiffusion in gel.

B. Thymoma Extract. The supernatant of the thymoma extract was heterogeneous on electrophoresis. It contained prealbumin, albumin, and globulin fractions. Both IgG and IgA immunoglobulins were demonstrated by immunodiffusion in gel.

II. Effect of Plasma and Fractions Thereof on Both in Vivo and in Vitro Systems

A. Effect of the IgG Fraction of Plasma Collected from a Series of Patients with Erythroblastopenia on in Vivo Erythropoiesis. Erythropoiesis induced by erythropoietin was not inhibited by normal IgG fractions, which were tested in at least 15 different assays. This was also true of normal

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Table 1
Effect of plasma and fractions thereof collected from patients with idiopathic erythroblastopenia on nucleoprotein and heme synthesis in vitro

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>% plasma*</th>
<th>Whole plasma erythropoietin units/ml</th>
<th>Erythropoietin units/ culture</th>
<th>% of control</th>
<th>No. in Chart 2</th>
<th>Comments</th>
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<td>M</td>
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<td></td>
<td></td>
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<td>&gt;0.7</td>
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<td>89</td>
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<td>&gt;3.3</td>
<td>&gt;0.7</td>
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<td>0.32</td>
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<td>2.5 mg/100 ml</td>
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* Of 2 ml bone marrow suspensions.
IgG Inhibitor in Plasma of Erythroblastopenic Patients

Table 2

Effect of plasma from patients with erythroblastopenia associated with lymphomatous disease on nucleoprotein and heme synthesis in vitro

<table>
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<th>Case</th>
<th>Sex</th>
<th>% plasma&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Whole plasma erythropoietin units/ml</th>
<th>Erythropoietin units/culture</th>
<th>% of control</th>
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<td>71</td>
<td>70</td>
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<sup>a</sup> Of 2 ml bone marrow suspension.

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Chart 1. Separation of whole plasma on Sephadex G-100 in 0.1 M ammonium bicarbonate buffer at pH 8.

plasma void-volume fractions collected from Sephadex G-100 columns. Inhibition of the incorporation of radioiron into erythrocytes by the IgG fraction of the plasma of 5 of 6 patients with a variety of erythroblastopenias in this series is shown in Chart 2. This was associated with a parallel decrease of the reticulocyte count. Two others, studied in more detail, also showed the inhibitory effect. Consequently, 8 of the 10 patients with erythroblastopenia demonstrated this effect in vivo.

B. Effect of Plasma from Patients with "Idiopathic" Erythroblastopenia on Nucleoprotein and Heme Synthesis in Vitro. Neither normal plasma nor extracts thereof demonstrated inhibition of nucleoprotein or heme synthesis in vitro (Table 1). The concentration of erythropoietin in the plasma of patients with idiopathic erythroblastopenia and anemia was extremely high. After human bone marrow cells were cultured with their whole plasma for 24 hr, RNA synthesis was significantly increased in 2 of 6 patients, and values were normal in the other 4 patients. When whole plasma was used, DNA and protein synthesis was significantly decreased in 2 of 6 of these patients. On the other hand, heme synthesis was significantly decreased in all patients in whom it was studied. The plasma of patients with sideroblastic and aplastic anemia showed no inhibition of either heme or nucleoprotein synthesis. Separation of the immunoglobulin from the erythropoietin in the plasma of Patient A., whose whole plasma did not have an in vitro inhibitory effect, resulted in a decrease of DNA synthesis when the IgG was added to the culture (see below). Increasing the concentration of IgG from the plasma of Patient M in the culture progressively decreased heme synthesis.

C. Effect of Plasma from Patients with Erythroblastopenia Associated with Lymphoma or Thymoma on Nucleoprotein and Heme Synthesis in Vitro. These patients (with the exception of Patient G), although significantly anemic, had much lower concentrations of erythropoietin in their plasma than expected. The higher percentage concentrat-
tion of whole plasma used in culture enhanced nucleoprotein and protein synthesis in those patients with lymphomatous disease. Reduction of the concentration of plasma and, consequently, the concentration of erythropoietin, unmasked the inhibitor of DNA, protein, and heme synthesis. Both patients with thymoma had depression of nucleoprotein synthesis at high concentrations of plasma, probably related to the significantly lower concentration of erythropoietin in their plasma. Dependence on the amount of IgG present was even more obvious when different concentrations of the IgG fraction of plasma were used instead of whole plasma (Patient J). Heme synthesis was decreased after incubation of bone marrow cells with whole plasma from all of the patients studied and was dose dependent when the extracted IgG of plasma of at least 1 patient (J) was studied in detail (Table 2).

**D. Correlation of In Vivo and In Vitro Data.** In addition to Patients, A. B., L, and J, whose results are discussed in greater detail below, it was apparent that when the IgG extracted from the whole plasma was used in the *in vivo* system, 5 to 6 patients inhibited the induction of *in vivo* erythropoiesis. In the sample (Patient R. M.) that did not inhibit erythropoiesis *in vivo*, nucleoprotein synthesis was unaffected, but there was some decrease of heme synthesis *in vitro*.

In all other samples demonstrating *in vivo* inhibition of erythropoiesis, those tested *in vitro* demonstrated inhibition of heme synthesis. When either samples of whole plasma or the IgG fractions were tested, 6 of the 10 patients showed inhibition of nucleoprotein synthesis when the optimal conditions were achieved. The most constant finding was a decrease of DNA and protein synthesis, although RNA synthesis was decreased in the 2 patients with thymoma. Thus the *in vitro* data appeared to correlate consistently with that obtained *in vivo*.

**E. Analysis of the Factors Influencing Assay Systems and In Depth Studies in 2 Patients with Erythroblastopenia and Thymoma.** 1. Patient L developed erythroblastopenia following thymectomy for thymoma. This illustrates the difficulties encountered with both the *in vivo* and *in vitro* systems when attempting to demonstrate inhibitory factors in the plasma. His plasma erythropoietin content was 0.1 unit/ml, which is lower than that usually found in these anemic patients. The results of his studies point out the need to use a correct ratio of erythropoietin to IgG inhibitor in the *in vivo* system before reporting negative results (Chart 3). When the DEAE-cellulose-eluted IgG fraction of his plasma was initially studied, a very large dose of erythropoietin was used in relation to the dose of IgG, and the results were not considered significant. When an optimum erythropoietin-to-IgG ratio was found by trial and error, the inhibitory effect of the IgG was clearly demonstrated. Incubation of the patient's erythropoietin with his IgG fraction *in vitro* did not reduce erythropoietic activity when tested in polycythemic mice. There was no precipitant line, on immunodiffusion in gel, when his erythropoietin was tested against his IgG fraction.

*In vitro*, his plasma inhibited nucleoprotein synthesis at a high plasma concentration, compared with that of the same concentration of normal plasma. Heme synthesis was also inhibited *in vitro* (Table 2).

2. In Patient J, a thymoma was removed, and the plasma was collected within the same period of time. His bone marrow showed erythroblastopenia. His plasma contained 0.5 unit erythropoietin/ml plasma (Table 2). The void volume of the Sephadex column and the IgG eluted from DEAE-cellulose produced marked inhibition of erythropoietin-induced erythropoiesis in polycythemic mice (Chart 4). The thymoma extract also proved to be a potent inhibitor of erythropoiesis. Incubation of erythropoietin with a fraction of the void volume of the Sephadex G-100 column resulted in a slight decrease of its erythropoietic effect in polycythemic mice. No precipitant line was formed when erythropoietin was tested against thymoma extract or the patient's IgG fraction by immunodiffusion in gel.

In the presence of a concentration of 0.25 unit of erythropoietin in bone marrow culture, the incorporation of tritiated thymidine, uridine, and leucine was still significantly decreased, as was heme synthesis (Table 2). Thymic extract completely abolished nucleoprotein synthesis. A dose-response effect of the IgG was observed *in vitro* for
both DNA and heme synthesis (Chart 5). This dose-response effect was also observed in the in vivo system (Chart 6).

**F. Effect of Various Plasma Fractions of a Patient with Idiopathic Erythroblastopenia on in Vivo Erythropoiesis and in Vitro Nucleoprotein Synthesis.** The plasma concentration of erythropoietin in Patient A. B. was high (Table 1). In polycythemic mice, erythropoiesis was inhibited by both the void volume fraction (78% inhibition) and the DEAE-cellulose-eluted IgG (84% inhibition). Incubation of the patient's IgG and erythropoietin did not affect erythropoietic activity, as measured in the polycythemic mouse assay (Chart 7). Addition of whole plasma to bone marrow cultures, which resulted in a concentration of 0.32 unit of erythropoietin in the culture, did not stimulate DNA synthesis, nor was there inhibition of nucleoprotein synthesis (Chart 8). However, addition of the fraction of the void volume of the Sephadex column significantly inhibited DNA synthesis by 50%, as did the IgG fraction. Indirect immunofluorescent technique employing γ-globulin from the patient's plasma was carried out by Dr. Ian Watson. He was unable to demonstrate any specific immunofluorescence of erythroblast nuclei. On immunodiffusion in gel, the patient's erythropoietin and IgG fraction failed to form a precipitant line. This patient also failed to respond to immunosuppression by 6-mercaptopurine.

**DISCUSSION**

Erythroblastopenia and its often curious association with thymoma have been the object of research by several investigators over the past few years. Early attempts to demonstrate inhibitors in biological fluids were unrewarding because of the lack of sensitivity and lack of specific methods by which one could detect these inhibitors in either tumor extract or plasma collected from these patients. By the use of the polycythemic mouse assay in which erythropoiesis can be suppressed and then induced by injection of erythropoietin, a particular substance that can either enhance or inhibit erythropoiesis can be studied in relation to induction of erythroblast formation. An important technical point arose when unfractionated whole plasma samples were used (18). Since these patients are often very severely anemic, their erythropoietin concentrations in plasma are often extremely high, as shown in these experiments. The absence of erythroid precursors in their marrow results in the failure of large concentrations of erythropoietin to induce erythropoiesis. Because of the high concentration of plasma erythropoietin, when whole plasma is tested in the in vivo system in which a normal number of erythropoietin-responsive cells are present, the stimulatory capacity of the sample appears to mask the presence of the inhibitor. This difficulty was overcome by separating the erythropoietin from the larger-molecular-weight inhibitor (IgG) from the plasma sample by molecular-weight sieve chromatography or by specifically extracting the IgG fraction on DEAE-cellulose. The former method could offer some advantages,
since larger immunoglobulins (IgM) could also be involved in the induction of erythroblastopenia (3, 5). The 2nd technical point is the use of a proper ratio of erythropoietin to IgG when studying the effect of the latter on erythropoiesis. If the amount of erythropoietin is excessive, as shown by the present data, the effect of the inhibitor can be masked. As the erythropoietin:IgG ratio approaches the optimum range, the effect of inhibition then becomes clearly apparent.

The fact that erythropoietin in the plasma of these patients can be separated by chromatography at a pH of 8 suggests that it is not bound in an antigen-antibody complex since, to dissociate antigen-antibody complexes, it is necessary to decrease the pH to 3 (34). This was not done because at this pH most of the biological activity of erythropoietin is known to be lost. Furthermore, when the erythropoietin and IgG of these patients were incubated and the supernatant was tested for erythropoietic activity, there was no significant reduction of erythropoietic activity in 2 patients and only a slight reduction of activity in 1, which was not considered to be of biological significance. This indicates that the inhibitor was not an anti-erythropoietin, as was thought possible in the initial study reported in 1966 (20), but was most probably directed against erythroid precursors. In the present series, 8 of 10 of the patients with erythroblastopenia demonstrated inhibition of erythropoiesis in vivo.

Of further interest was the recovery of more units of erythropoietin from the Sephadex G-100 column than calculated from the whole plasma content of patients with thymoma. While this often occurs when normal plasma with high erythropoietin content is fractionated, there appeared to be a disproportionately greater recovery. This suggests that more erythropoietin was present in the plasma than was measured due to the presence of the inhibitor.

The in vitro data further delineated the direct effect of the IgG fraction upon hemopoiesis. Whole plasma containing high concentrations of erythropoietin collected from patients with idiopathic erythroblastopenia significantly decreased DNA synthesis at 24 hr in only 2 of 5 patients. In both positive samples there was an associated decrease of protein synthesis. Heme synthesis was decreased after 24 hr of incubation in all patients studied. When the IgG was added, devoid of erythropoietin, the inhibition of DNA synthesis was readily demonstrated. The lack of effect on nucleoprotein synthesis when whole plasma was used is viewed as being due to the presence of excessive erythropoietin.

In those patients with erythroblastopenia associated with lymphomatous disease when a relatively high concentration of plasma was used, inhibition of DNA synthesis was induced only by the plasma of those patients who had a low concentration of erythropoietin. However, as the percentage plasma concentration in the culture was decreased and concomitantly the erythropoietin concentration, or the extracted IgG alone was added, inhibition of DNA synthesis was apparent. There was also, at least in 1 patient of this group, a dose-response effect upon the addition of increasing amounts of isolated IgG to the culture. RNA synthesis was inhibited in only 2 patients, both of whom had a thymoma. In all the patients studied in this group, heme synthesis was decreased. The effect was amplified by reduction of the amount of erythropoietin in the culture and by increasing the concentration of the amount of pure IgG added to the culture. In no case was heme synthesis shown to be increased after bone marrow cells were cultured in the presence of rather high titers of plasma erythropoietin.

It has previously been shown that increased heme synthesis in vitro occurs 10 hr later upon addition of erythropoietin to the culture (23). The inhibition of heme synthesis at 24 hr suggests that the erythroid precursors were damaged by the IgG present in the plasma of these subjects. Since the clinical picture is that of the absence or marked reduction of erythroblasts and later precursors in the bone marrow, this could be due to the failure of differentiation because of the destruction of proerythroblast precursors as suggested by Field et al. (10) or, alternatively, could be due to destruction of erythroblast and early erythroid precursors in situ as previously suggested (21). Although antinuclear antibodies have been described in this disease (4, 24), it remains to be determined whether such antibodies are physiologically active in the production of the disease process or are secondary to a cytotoxic effect, e.g., damage to the cell membrane by antibody. The present studies indicate that nucleoprotein synthesis is inhibited in at least some of the cases, more specifically those associated with tumor, but the data do not indicate whether this is a primary or secondary effect. Recently, it has been shown that in a cell-free system, antinuclear antibodies failed to interfere with DNA template activity (36). This information suggests that the suppression of the synthesis of DNA was not the primary factor that induced the pathological disease known to be associated with antinuclear antibodies, although this effect cannot be totally ruled out. It is the cell surface that is the main mediator between the cell environment and its internal milieu. The clinical observations of absence of erythroid precursors and the present data which show a consistent decrease of DNA, protein, and heme synthesis in culture suggest that the circulating factor is cytotoxic (21), resulting in cell destruction with secondary depression of heme synthesis and nucleoprotein synthesis. Indeed, this interpretation of the results would appear to be supported by the recent demonstration of a factor in the plasma of a patient with erythroblastopenia which induces release of labeled membrane substance into the culture medium following incubation of bone marrow cells with the plasma substance (37). Others have shown that sera from patients with thymoma and erythroblastopenia can inhibit the proliferation and maturation of normal human bone marrow in culture and, by means of tagging immunoglobulins with fluorescent dye, it was shown that an IgA and IgM were specifically bound by the bone marrow cells (3, 5). It then follows that the nature of surface antigens present on early erythroblasts must be studied to determine whether these cells contain antigens not found on mature erythrocytes, since the latter did not have incomplete antibody directed against the mature erythrocytes found in their serum.

The potent inhibitory effect of thymoma extract observed
in these experiments must be viewed cautiously, since normal thymus tissue was not available for testing. It could not be directly related to a specific substance since the thymic extract was a very heterogeneous material. Both IgG and IgA were present in the extract, and the inhibitory effect could well have been related to extraction of immunoglobulin from trapped plasma within the tumor. On the other hand, the thymoma tissue could well have contained a specific substance that inhibits erythropoiesis both in vivo and in vitro. This possibility is presently under investigation.

Since thymomas have been found in approximately 50% of cases of erythroblastopenia, a meaningful relationship between these 2 entities is strongly suggested. The exact mechanisms (8) are obscure. Consequently, it is not surprising that the induction of erythroblastopenia is a result of cytotoxic damage to erythroid precursors. However, the initiation of the primary immune process remains obscure, as does the primary or associated relationship to thymoma and other types of lymphomatous disease observed in this small series. The problem is clearly not solved, and the possible role of viral-induced damaged cells which could result in release of antigenic cell membrane, direct alteration of nucleoprotein synthesis by virus, or the effect of cell-mediated immune processes must be investigated.

The present series of 10 patients lends further support to what has previously been reported (2, 3, 5, 6, 14, 18, 29, 37), that is, that a circulatory inhibitor, presumably an immunoglobulin in most cases, can under the proper conditions be consistently demonstrated in these patients either by in vivo or in vitro methods.

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Decreased *in Vivo* and *in Vitro* Erythropoiesis Induced by Plasma of Ten Patients with Thymoma, Lymphosarcoma, or Idiopathic Erythroblastopenia

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