Studies on Red Cell Aplasia. III. Treatment With Horse Antihuman Thymocyte Gamma Globulin

By Sanford B. Krantz

A case of pure red cell aplasia, which did not respond to corticosteroids, splenectomy, or cyclophosphamide, has been described. In vitro studies performed with the patient's marrow showed a much smaller response to erythropoietin than that observed with previous patients who did respond to cyclophosphamide. The patient's plasma and γG-globulin fraction inhibited the rate of heme synthesis of normal marrow cells in vitro. The γM-globulins did not inhibit heme synthesis in the same system. The patient was treated with cyclophosphamide and horse antihuman thymocyte gamma globulin (HAHTGG) and had a definite increase in red cell production, but a severe febrile reaction occurred that prevented further use of the HAHTGG. This study indicates that HAHTGG may be of aid in treating severe cases of red cell aplasia when more effective means of minimizing hypersensitivity reactions are devised.

THREE CASES OF PURE RED CELL APLASIA in adults have been previously reported in which a remission of the disease was produced by immunosuppressive therapy.1-3 All three patients had bone marrows that were virtually devoid of red cell precursors, but when the marrows were incubated in vitro for 50–70 hr the rate of heme synthesis increased beyond the initial rate. In addition, the cultured marrow cells responded to erythropoietin with a further increase in the rate of heme synthesis. In one case, the patient's plasma inhibited the increase in the rate of heme synthesis by his marrow.1 In another case, the patient's γG-globulins, obtained prior to treatment, inhibited heme synthesis of normal marrow cells, while the γG-globulins obtained after remission of the disease did not.2 In the third case, a plasma inhibitor of heme synthesis was not identified.3 In all three cases a γG-globulin antibody to erythroblast nuclei was found. These studies suggested that pure red cell aplasia might be due to an antibody to the marrow erythroblasts that either prevented their development or destroyed them as they were formed. Some cases of pure red cell aplasia were treated subsequently with a similar course of immunosuppressive drugs but did not have a remission of the disease.4 While some of these cases could represent a different pathogenetic mechanism, others may be a more severe form of the same disease process. Immunosuppressive therapy may not always be successful because of...
the onset of drug-induced marrow toxicity before adequate immunosuppression is produced. This paper describes a case of pure red cell aplasia that did not respond to cyclophosphamide but appeared to be due to the same mechanism as the previous three cases. Subsequently, horse antihuman thymocyte gamma globulin (HAHTGG) was administered, which produced a definite increase in red cell production, but had to be stopped because of a severe febrile reaction.

CASE REPORT

R. B., a 59-yr-old white female, was in good health until 1962 when she developed urticaria. The patient was not taking medications at that time and did not recall any contact with insecticides or toxic chemicals. Soon afterward, she was noted to be anemic, and a diagnosis of pure red cell aplasia was made based on an absence of reticulocytes and marrow erythroblasts in an otherwise normal marrow with normal white cell and platelet counts. The patient was treated with large amounts of corticosteroids without a therapeutic effect. She developed multiple rib fractures, cataracts, and a bleeding peptic ulcer that necessitated a partial gastrectomy. A splenectomy was also performed, but the splenic morphology was unremarkable and this did not appear to increase her erythropoiesis. She was maintained on red cell transfusions requiring approximately 1 U/wk and she developed hemosiderosis with hepatomegaly, diabetes mellitus, and cardiac arrhythmias.

In June, 1966, the patient's physician sent some of her fresh frozen plasma to this laboratory for erythropoietin and antibody studies. The plasma erythropoietin level was found to be markedly elevated using the polycythemic mouse assay. The γG-globulins were extracted by previously described methods and a portion was conjugated with fluorescein isothiocyanate. When the γG-globulins were added to normal marrow cells, a marked inhibition of heme synthesis occurred. An antibody to erythroblast nuclei was demonstrated, but the patient also had antibody to the nuclei of peripheral blood lymphocytes. Review of a recent marrow biopsy confirmed the diagnosis of pure red aplasia.

As a result of these studies, the patient's physician treated her with cyclophosphamide, up to 200 mg/day for 32 days, until her white cell count had declined to 1200 cells/cu mm. She may have had a slight decrease in her transfusion requirement, but no reticulocytosis was noted. The cyclophosphamide was reinstituted several weeks later, and although it was administered for 2 mo at 100-150 mg/day, no remission of the disease occurred.

In August, 1970, the patient was referred to the Vanderbilt University Clinical Research Center. Physical examination revealed a short, asthenic woman who appeared wasted and chronically ill. The principal physical findings were cardiomegaly, marked hepatomegaly, and a dark, atrophic skin. Laboratory tests revealed 0.1% reticulocytes and a white cell count of 8100 cells/cu mm. The patient had been recently transfused and was not anemic. The platelet count, which had been normal throughout her illness, was 42,000 cells/cu mm. Serum folate and B₁₂ levels were normal, as was the Schilling test for vitamin B₁₂ absorption, but the fecal fat was 8.8 g/day. The serum iron was 122 μg/100 ml, and the total iron binding capacity was 144 μg/100 ml. The blood glucose was 270 mg/100 ml. Histoplasmin, tuberculin PPD (second strength), and mumps skin tests were negative. A skin test to Candida albicans was weakly positive. In vitro lymphocyte transformation tests, performed by Dr. Robert Alford, showed a normal response to phytohemagglutinin but no response to histoplasmin or tuberculin antigens. Serum complement activity was estimated through measurement of C₃ by the radial immunodiffusion technique (Hyland, Los Angeles, Calif.) and was low at 81 mg/100 ml. Immunoglobulin assays were performed using the immunodiffusion technique (Hyland, Los Angeles, Calif.). The γG-globulin concentration was 1312 mg/100 ml; the γA-globulin concentration was 352 mg/100 ml; and the γM-globulin concentration was 122 mg/100 ml. Immunoelectrophoresis of the patient's serum revealed no abnormal paraproteins. Roentgenograms of the chest showed
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only cardiomegaly. In vitro studies of the patient's marrow were begun at this time. The protocol for this study was approved by the Vanderbilt University Committee on Clinical Investigation. Informed consent was obtained from the patient before study.

MATERIALS AND METHODS

Bone marrow cell cultures were prepared as previously described,8,9 by passing the marrow through a pipette several times to disperse the cells, and then washing the cells four times with 5 ml of Hanks' balanced salt solution to remove the patient's plasma and the marrow fat. The cells were diluted with an aliquot of NCTC-109 (Microbiological Associates, Bethesda, Md.) and were pipetted into 35 x 10 mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) to which heparinized human plasma or sterile-filtered, precooled straw, newborn calf serum (Colorado Serum, Denver, Colo.) had been added. Heparin, penicillin, and streptomycin were added to produce final concentration of 4 u/ml, 40 u/ml, and 2 ug/ml, respectively. 59FeCl3 was attached to transferrin by incubation with a mixture of 60% NCTC-109 and 40% human type AB plasma, at 37#{176}C overnight, and 0.1 ml of this solution was added to the cultures at later intervals. Radioactive heme was extracted and counted as in the previous reports. A Low Beta (Beckman, Fullerton, Calif.) gas flow counter with a background of 1.0-1.5 cpm was used. Sheep plasma erythropoietin concentrate, 3.3 U/mg of protein (Step III, Lot K 147 187) was obtained from the U. S. Public Health Service Study Section on Hematology. Human urinary erythropoietin concentrate, 65 U/mg of protein (Belcher pool H) was obtained from Dr. Frederick Stohlman, Jr. The normal horse gamma globulin (lot 16,229) and HAHTG (lot 16,138-2) were provided by Upjohn, Kalamazoo, Mich. The HAHTGG had a cytotoxic titer of 1:65,536 and hemagglutination titer of 1:1024.10 The rosette inhibition titer determined by the procedure of Parcelles and Gray11 was 1:56,400.10

The gamma-globulins were extracted from the plasma and purified by ammonium sulfate precipitation12 and DEAE-cellulose column chromatography.13 All glassware and columns were previously cleaned and prepared for tissue culture as described by Paul.14 The purified gamma-globulins were concentrated by ultrafiltration with collodion bags (Schleicher & Schuell, Keene, N. H.) that had a 25,000 mo/wt pore size and had been soaked 24 hr in 7X (Linbro Chemical, New Haven, Conn.), 24 hr in a sodium metasilicate (0.1 g/100 ml) and Calgon (0.01 g/100 ml) solution, and 24 hr at pH 2.8 N HCl, before being rinsed with distilled water. The gamma-globulins were then placed in dialysis tubing that had been similarly prepared and were dialyzed for 14 hr in 200 volumes of NCTC-109 (Difco, Detroit, Mich.) with 80 U of penicillin and 50 ug of streptomycin per ml, followed by dialysis of 8 hr in 50 volumes of NCTC-109 (Microbiological Associates) with the same concentrations of penicillin and streptomycin. The gamma-globulins were sterilized by filtration through Swinnex-25 filter units (Millipore, Bedford, Mass.) with a 0.45 u pore size and were stored at —80#{176}C. Ten milliliters of Hanks' solution were passed through the filters before they were used to sterilize the gamma-globulins. All protein concentration were measured by the ratio of absorbance at 260 and 280 nm.15 Immunoelectrophoresis was performed on ion-agar plates in 0.025 M Veronal buffer, using goat polyvalent antihuman serum (Hyland), as well as rabbit antisemur (Calbiochem, Los Angeles, Calif.) specific for human gamma-globulins.

Ultracentrifugation of plasmas was performed at 25,000 rpm for 24 hr in a Beckman SW 25.1 swinging bucket rotor. Sucrose was dissolved in Hanks' solution at concentrations of 10%, 20%, 30%, and 40%. Cellulose nitrate tubes for this rotor were washed with 100% ethanol and air dried before layering 6.6 ml of each of these sucrose solutions in the tubes, beginning with the 40% solution. The tubes were stored at 3#{176}C for 24 hr to establish a gradient, and 2.2 ml of plasma, to which 4.4 ml of Hanks' solution had been added, was layered on top of the sucrose gradient. After centrifugation, 1-ml fractions were collected by aspiration from the top of the tubes, and the absorbance at 280 nm was determined. A small aliquot of these fractions was tested for gamma-g-, gamma-A-, and gamma-M-globulins by the Ouchterlonny immunodiffusion method using specific antisera (Hyland). On the basis of the Ouchterlonny determinations, the 1-ml fractions were pooled into three groups.
Fig. 1. Effect of erythropoietin on marrow of patient with red cell aplasia. Lower part of figure represents two separate experiments in which cultures were started with 950 nucleated cells/cu mm in total volume of 0.8 ml. Cultures had 20% normal human plasma, 20% newborn calf serum, and 60% NCTC-109. Controls (black circle and black square) had no added erythropoietin; stimulated cultures (open circle and open square) had 0.6 U/ml of urinary erythropoietin. Each point indicates middle of a 17-hr incubation with 1.6 μCi ³²Fe and represents mean of four cultures plus SEM. Upper part of figure represents experiment in which cultures were started with 900 nucleated cells/cu mm in total volume of 0.9 ml with 44% of patient's plasma and 56% Hanks' solution. Each point indicates middle of a 20-hr incubation with 1.2 μCi ³²Fe and represents mean of six cultures.

These were concentrated by ultrafiltration to 2.2 ml and dialyzed twice for 24 hr against 100 volumes of NCTC-109 (Microbiological Associates) with 50 U of penicillin and 5 μg of streptomycin per ml. The ultrafiltration bags and dialysis tubing were prepared for tissue culture by the methods described above.

Normal horse gamma globulin was centrifuged at 14,500 g for 1 hr. The top 5% was removed and administered intravenously within 30 min through a Millipore 0.45 μm filter. Analytical ultracentrifugation of this material was performed at a concentration of 26 mg/ml using the Beckman model E centrifuge with a sector cell and schlieren optics. During a period of 40 min at 56,000 rpm only the presence of one symmetric peak was present, indicating that the gamma globulin was free of detectable aggregates. The horse antihuman thymocyte gamma globulin was administered in 250 ml of normal saline with 3000 U of heparin over 4 hr with a Millipore 0.45 μm filter attached in the intravenous tubing.

RESULTS

When the patient's marrow cells were treated with erythropoietin in vitro, the rate of heme synthesis increased above the initial rate (Fig. 1). This increase was statistically significant (p < 0.01), but was far less than the increases previously observed using the same erythropoietin at the same dose or a different preparation at one-half the dose. Without the addition of
erythropoietin the rate of heme synthesis fell below the initial rate (Fig. 1), whereas previously this rate had increased, although to a lesser extent than when erythropoietin was added.1-3

Increasing concentrations of the patient’s plasma, normal plasma, or the plasma of a patient with primary refractory anemia were incubated with the patient’s marrow, and the rate of heme synthesis was measured after 46 hr (Fig. 2). The rate of heme synthesis markedly increased with high concentrations of normal plasma, or the plasma from the anemic patient. High concentrations of the patient’s plasma prevented this increase in the rate of heme synthesis by the patient’s marrow.

At a later time, while the patient was being treated with cyclophosphamide and prednisone, her marrow cells were incubated with Hanks’ balanced salt solution and her own plasma without the addition of normal human plasma or calf serum (Fig. 1). An increase in the rate of heme synthesis was observed \(p < 0.01\) when the only new ingredients with which the marrow had contact were heparin and antibiotics. No new nutrients were present that could have caused this effect.

\[
\text{HEME} \times 10^6 \times 10^{-3} \text{C.P.M.} \times \text{MEDIUM Fe (\mu g)}
\]

**Fig. 2.** Effect of patient’s plasma on heme synthesis by patient’s marrow. Cultures were begun with 900 nucleated cells/cu mm in medium of 11% Hanks’ solution and either patient’s plasma (black triangles), normal plasma (black circles), or plasma from a patient with primary refractory anemia (black square). NCTC-109 was added to make total volume of 0.9 ml. All cultures had 0.13 U/ml of sheep erythropoietin. \(^{59}\)Fe (2.0 mCi) was added after 46 hr of incubation for period of 24 hr before terminating experiment. Each point represents mean of four cultures plus SEM. Heme cpm was multiplied by total amount of iron present in medium of each culture to correct for variations of unlabeled iron in the plasmas.
Fig. 3. Effect of patient’s purified γG-globulins on heme synthesis by normal marrow. Cultures were begun with 1750 nucleated cells/cu mm in total volume of 1.1 ml with 0.4 ml normal plasma, 0.1 ml Hanks' solution, and 0.6 ml NCTC-109, which contained γG-globulins extracted from patient’s plasma (dashed line) or normal plasma (solid line). After 40 hr of incubation 0.9 μCi of 59Fe was added for 24 hr before terminating cultures. Each point represents mean of three cultures plus SEM. All cultures had 0.11 U/ml of sheep erythropoietin.

Table 1. Effect of Patient’s Plasma on Heme Synthesis by Normal Marrow

<table>
<thead>
<tr>
<th>Patient’s Plasma (%)</th>
<th>Normal Plasma (%)</th>
<th>Heme 59Fe x Medium Fe (cpm) x (μg)</th>
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<tbody>
<tr>
<td>0</td>
<td>88.8</td>
<td>351 ± 11.1*</td>
</tr>
<tr>
<td>11.1</td>
<td>77.7</td>
<td>88.2 ± 1.6</td>
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<tr>
<td>33.3</td>
<td>55.5</td>
<td>102 ± 7.2</td>
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<td>55.5</td>
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<tr>
<td>80.0</td>
<td>10.0</td>
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</tr>
<tr>
<td>88.8</td>
<td>0</td>
<td>70.0 ± 24.2</td>
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</table>

*Standard error of the mean.

Cultures were begun with 1500 nucleated cells/cu mm in a total volume of 0.9 ml, with 0.8 ml plasma and 0.1 ml Hanks' solution. All cultures had 0.13 U/ml of sheep erythropoietin, 0.55 U/ml of heparin, 0.55 U/ml of penicillin, and 0.3 μg/ml of streptomycin. After 43 hr of incubation, 1.1 μCi of 59Fe was added for 24 hr before terminating cultures. Heme cpm was multiplied by total amount of iron present in medium of each culture to correct for variations of unlabeled iron in plasmas. Each product represents mean of three cultures.
Fig. 4. Effect of patient’s γM-globulins on heme synthesis by normal marrow. Fractionation of patient’s plasma (A) and normal plasma (B) was performed by sucrose gradient centrifugation as described in text. After absorbance (black circles) was measured and presence of IgG, IgA, and IgM (solid line) was determined, the fractions were pooled into three groups. These were concentrated to original plasma volume, dialyzed in NCTC-109, and tested with normal marrow cells in vitro for an effect on heme synthesis (solid bars). Each bar represents mean of four (A) or three (B) cultures plus SEM. The heme cpm was multiplied by total amount of iron present in medium of each culture to correct for variations of unlabeled iron in different pools. Cultures were begun with 100 nucleated cells/cu mm in total volume of 1.1 ml with 0.2 ml normal human plasma, 0.2 ml calf serum, 0.1 ml Hanks’ solution, and 0.6 ml NCTC-109 that contained pool 1, 2, or 3. After 45 hr of incubation, 1.5 μCi of 59Fe was added for 24 hr before terminating cultures. All cultures had 0.22 U/ml of urinary erythropoietin, 108 U/ml of heparin, 32 U/ml of penicillin, and 1.6 μg/ml of streptomycin.

The patient’s plasma was tested for an inhibitor to heme synthesis by diluting it with increasing concentrations of normal plasma and incubating the mixtures with a normal marrow (Table 1). Increasing amounts of normal plasma did not increase the rate of heme synthesis, as long as some of the patient’s plasma was present. As little as 11.1% of the patient’s plasma greatly inhibited the rate of heme synthesis.

The patient’s γG-globulins and γG-globulins from normal plasma were purified at the same time. Increasing concentrations of these preparations were added to normal marrow cells in vitro and the rate of heme synthesis was measured after 48 hr (Fig. 3). The patient’s γG-globulins markedly inhibited the rate of heme synthesis compared to normal γG-globulins.
In order to test the \( \gamma \)-globulins for a capacity to inhibit heme synthesis, the patient’s plasma and normal plasma were layered on sucrose gradients and centrifuged to separate the \( \gamma \)-globulins from the bulk of the \( \gamma \)G- and \( \gamma \)A-globulins. One milliliter aliquots were collected, and the absorbance at 280 m\( \mu \) was measured (Fig. 4). The presence of \( \gamma \)M-, \( \gamma \)G-, and \( \gamma \)A-globulins in each of the aliquots was determined by Ouchterlony immunodiffusion. The aliquots were then combined into three main pools with the \( \gamma \)M-globulins present in only the second pool. These pools were incubated with normal marrow cells, and the effect on the rate of heme synthesis was measured. The \( \gamma \)M-globulin pool did not inhibit the rate of heme synthesis. Only the pool with the bulk of the \( \gamma \)G-globulins in it demonstrated a capacity to inhibit. Thus the \( \gamma \)M-globulins did not appear to participate in the inhibition of heme synthesis by the patient’s plasma.

Since this patient appeared to be similar to previous patients who had responded to immunosuppressive drugs and since this patient had failed to respond to cyclophosphamide alone, she was subsequently treated with a combination of cyclophosphamide and HAHTGG (Fig. 5). Prior to this treatment, the patient had no erythroblasts in the blood and virtually none in the marrow. Her reticulocyte count varied from 0% to 0.3% despite a severe anemia. A skin test with 7.5 mg of normal horse gamma globulin showed no reaction over 24 hr, and cyclophosphamide was started using the same schedule previously followed by the patient’s doctor. Two days later, 6 mg of aggregate-free normal horse gamma globulin was administered intraven-
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Continuously to induce tolerance to the HAHTGG.\textsuperscript{17} No adverse reactions were noted. This was repeated the following day, and 24 hr later HAHTGG was begun at 500 mg/day. Halfway through the initial infusion, the patient developed a chill and later a high fever of 40°C. She became hypotensive, but the fever and hypotension subsided after aspirin therapy. She also had a moderate amount of hemolysis, as indicated by a 25% decrease in her hematocrit. The reaction to the HAHTGG appeared to be a direct toxic effect of the drug, since no manifestations of allergy or hypersensitivity such as urticaria or bronchospasm were apparent.

After 3 days, the patient felt well again, and the HAHTGG was begun at one-half the original dose. The patient developed chills and fever midway through each infusion, but this was easily controlled with aspirin and antihistamine, and she tolerated this dose of the medication without hypotension or a rapid decline in her hematocrit. The dose of HAHTGG was then gradually increased without further adverse side effects. A marked lymphocytopenia was produced, and because of a rapid decline in the white cell count by the 16th day of treatment, the cyclophosphamide was decreased slightly below the previous schedule for a short period of time (Fig. 5). A significant reticulocytosis, accompanied by erythroblasts in the peripheral blood, was noted by the 28th day of therapy. This became very evident during the following week when 0.8% reticulocytes and 700 erythroblasts/cu mm were present in the peripheral blood. However, several days after the onset of increased erythropoiesis the patient began to have fever that persisted following the infusion of HAHTGG and became nauseated and anorexic. Because of the possibility of infection, the HAHTGG and cyclophosphamide were discontinued. The fever persisted with daily peaks of 39–40°C, but frequent cultures revealed no bacterial or fungal organisms that might be responsible. On several occasions, localized edema of the upper eyelids was noted, but urticaria, arthralgias, asthma, or other clinical evidence of serum sickness were not present. Proteinuria or uremia did not occur during the period of febrile illness. The patient gradually defervesced, and the fever ended 3–4 wk after the onset. The patient’s condition at this time appeared to be the same as before the start of the HAHTGG, but reticulocytes and erythroblasts were no longer present in the blood.

Antibody titers against horse gamma globulin were performed by Dr. Karl Hubner using the passive hemagglutination technique of Avrameas et al.\textsuperscript{18} Antibody titers, which were zero prior to treatment, increased from 1:4 to 1:16 in the month following discontinuation of the HAHTGG. Complement (C3) determined by the immunodiffusion technique (Hyland) was low at the onset of treatment and remained low throughout the study period.

Two months after discontinuing the HAHTGG, readministration of this agent was considered. Intradermal injections of normal horse gamma globulin were begun every 15 min, starting with 0.0005 μg and increasing the concentration by a factor of ten with each injection.\textsuperscript{19} After the administration of 500 μg the skin showed a flare and then a wheal, and the patient experienced chest pain. Sublingual nitroglycerin and intravenous methylprednisolone were
administered, and the pain shortly disappeared. Because of a suspected allergic reaction to the horse protein no further therapy with this drug was attempted.

This patient was subsequently treated with a course of cyclophosphamide that was identical to the one accompanying HAHTGG. In place of the latter drug, she received 60 mg of prednisone per day. No blood reticulocyte or erythroblast response was observed. The cyclophosphamide and prednisone were continued for an additional month to the point of marrow toxicity as in previous patients and were then stopped. No hematologic response was observed within 4 wk after stopping the cyclophosphamide and tapering the prednisone to physiologic levels. At that time, the patient developed a gastric ulcer requiring surgery and she subsequently died postoperatively of infection with progressive liver and renal failure.

DISCUSSION

This patient had pure red cell aplasia as evidenced by a virtual absence of erythroblasts in the bone marrow and reticulocytes in the blood, with a normal marrow cellularity and normal granulopoiesis and megakarycytopoiesis. Her disease appeared to have a similar pathogenesis as the cases we have previously studied. The plasma erythropoietin concentration was very high. The marrow responded to erythropoietin in vitro and the rate of heme synthesis increased above its initial rate. When the marrow was cultured in a dilute solution of the patient's plasma, the rate of heme synthesis increased without the addition of new nutrients, suggesting that it had been freed from some inhibitory factor. The patient's plasma prevented a large increase in the rate of heme synthesis by her marrow that occurred when the marrow was incubated with normal plasma or the plasma of another chronically anemic and chronically transfused patient. The patient's plasma and purified γG-globulins inhibited the rate of heme synthesis of normal marrow. The patient's plasma had an antibody to erythroblast nuclei as determined by the previously described techniques, but antibody to blood lymphocyte nuclei was also present.

Although similar laboratory observations were made in this patient, she did not have a therapeutic response to cyclophosphamide alone. Since the pathogenesis of the disease appeared to be similar, the lack of response to cyclophosphamide probably was due to an increased severity of the same process. The severity seems to be reflected best in the response of the marrow to erythropoietin in vitro. When the marrows of previous patients with this disease were cultured in a similar mixture of normal human plasma, newborn calf serum, and NCTC-109, the rate of heme synthesis increased five to six times above its initial rate with the addition of erythropoietin. The increase in the rate of heme synthesis in this patient's marrow was less than twofold but was statistically significant. Further study with additional patients is of course necessary to make a significant correlation between the response of the marrow to erythropoietin in vitro and the degree of severity of the disease that would allow prognostication of response to immunosuppressive therapy. Of all the parameters studied up to date, however, the response of the marrow to erythropoietin in vitro has been the most consistent indicator.
of response to immunosuppressive treatment, and it is the test that is most capable of precise quantitation. When this in vitro response is inhibited by the patient’s plasma or γG-globulins, the full picture is apparent, but in at least one case, direct inhibition could not be demonstrated.³

The severity of this patient’s disease is also indicated by its long duration and lack of improvement with corticosteroids or splenectomy. In this situation, immunosuppressive therapy may not be successful because of the production of marrow toxicity before adequate immunosuppression occurs. The therapeutic to toxic ratios of current immunosuppressive drugs may not be great enough to treat the more severe cases. For this reason, additional agents must be found that can increase this ratio when added to our current drugs. Antilymphocyte globulin (ALG) is one of the most potent immunosuppressive drugs available,²⁰,²¹ and it has a minimum of marrow or systemic toxicity aside from allergic reactions to a foreign protein.²¹,²² This agent causes a depletion of the thymus-dependent lymphocytes of the peripheral blood and paracortical areas of lymph nodes, while sparing the lymphocytes of the lymph follicles and germinal centers.²¹,²²

When ALG was first studied, it was thought that it had a specific effect on cellular immunity alone.²¹ It soon became evident, however, that ALG inhibited the primary humoral antibody response in addition.²³–²⁶ Doses that were effective in suppressing the primary antibody response had little effect on the secondary antibody response.²⁴–²⁶ This difference, in effect on the primary and secondary humoral response, could be due to the fact that ALG destroys lymphocytes that are involved in the initiation of immunologic reactions.²³–²⁴,²⁶ It appears, however, that it is more likely due to the difference in accessibility of ALG to different lymphocytes rather than to the immune process with which the cell is involved.²⁴,²⁵ This is indicated by the experiments of James in which ALG was incubated in vitro with sensitized lymphocytes and destroyed their ability to restore the secondary humoral response.²⁵

From the work of these investigators it appears that ALG, by itself, may be most effective in those autoimmune diseases where circulating antibody does not play a major role.²⁷ However, our thought in administering it to a patient with red cell aplasia was not to use it as the principal immunosuppressive agent, but rather as an additional agent that might increase the therapeutic-to-toxic ratio of the combined immunosuppressive therapy. The fact that the patient did have a definite erythropoietic response to the combination of cyclophosphamide and HAHTGG when she did not respond to cyclophosphamide alone or cyclophosphamide and prednisone supports this hypothesis. Since ALG has relatively little marrow toxicity,²² it can be added to cyclophosphamide in large doses and, when given for long periods, it may reach a sufficient number of lymphocytes involved in the secondary humoral response to produce a successful therapeutic result. Alternatively, it is possible that a component of cellular immunity is involved in the pathogenesis of red cell aplasia and that combined treatment with HAHTGG and cyclophosphamide is more effective because it inhibits this component of the disease. At the present time, little is known about any role of cellular immunity in this disease,
and further studies are necessary to test this possibility.

While this study demonstrates that ALG may be of aid in the immuno-suppressive treatment of red cell aplasia and may act to increase red cell production, it also indicates that a more effective way of using this agent will have to be devised in order to prevent or minimize allergic reactions to the foreign protein. In this patient, aggregate-free normal horse gamma globulin was administered in an attempt to induce tolerance to the horse protein as recommended by Butler et al.\textsuperscript{17} Recent investigations have shown that aggregate-free normal gamma globulin administered in this manner may not create tolerance in some individuals but may in fact immunize them.\textsuperscript{28} However, Rossen et al.\textsuperscript{29} found deaggregated normal horse gamma globulin to be effective in preventing antibody formation to ALG, when it was combined with higher doses of prior chemical immunosuppressive therapy. This indicates that either normal horse gamma globulin should not be administered prior to ALG treatment, or it should be combined with greater amounts of chemical immunosuppressive drugs.

Gewurz et al.\textsuperscript{30} have demonstrated that infusion of large amounts of ALG (4–20 mg/kg) may induce immunologic tolerance in certain patients. Therefore, a better schedule of ALG administration would start with one-half dose (250 mg) to avoid the initial toxic reaction seen in this patient and proceed to the immediate institution of large ALG doses (500 mg) on succeeding days, rather than a slow gradual increase in the dose as given in this patient. Finally, ALG may be more effective in combination with other immunosuppressive drugs rather than cyclophosphamide alone. Corticosteroids were not administered with the ALG in this patient, but in the future it would seem desirable to combine steroids with cyclophosphamide and ALG, in order to arrive at the most effective and least toxic course of immunosuppressive therapy. By these changes, hypersensitivity reactions such as the one that occurred in this patient might be avoided and a better therapeutic outcome achieved.

It must be emphasized that HAHTGG is a new drug, and further studies are necessary to delineate its effectiveness in patients with red cell aplasia. Until a more effective schedule is devised and its possible benefit in these cases is clearly defined, study of HAHTGG in this disease should be reserved to patients with pure red cell aplasia who have some of the biochemical characteristics shown here and who have failed to respond to conventional immunosuppressive agents. In order to learn more about its effect, it should be administered as the only new agent added to a previous combination of immunosuppressive drugs. Despite its limitations, HAHTGG may prove to be a suitable agent for use in pure red cell aplasia, since prior experience with immunosuppressive drugs in this disease indicates that only a short treatment period of about 2 mo is generally necessary to induce a remission. Hopefully, these studies will lead to an effective means of treatment for those individuals who have a much more severe form of the disease, such as the patient described here.
ACKNOWLEDGMENT

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REFERENCES

23. Turk, J. L.: Pathological effects and


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