Variable in Vitro Erythropoiesis in Patients with Transient Erythroblastopenia of Childhood

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Transient erythroblastopenia of childhood (TEC) is a pure red cell aplasia which primarily affects children in the infant and toddler age group. The clinical syndrome of TEC is well defined and is characterized by moderate to severe anemia with reticulocytopenia, selective aplasia of the erythroid bone marrow elements, and spontaneous recovery, usually within a month of presentation. We utilized the plasma clot tissue culture technique to explore the defect of erythropoiesis in seven patients with TEC. Culture of bone marrow at diagnosis in four patients revealed an increased erythroid proliferative capacity in one and a decreased capacity in three. The former patient plus three additional patients were found to have a transient serum inhibitor of erythroid colony formation in autologous and allogeneic systems. The three patients with diminished erythroid proliferative capacity had no demonstrable serum inhibitor, and in one patient studied the erythroid proliferative capacity became supernormal after recovery. We conclude that although TEC has a characteristic clinical picture, in vitro studies reveal a variable expression of the erythropoietic defect and support the hypothesis of a heterogeneous pathogenesis of this disorder.

Transient erythroblastopenia of childhood (TEC) is a syndrome characterized by temporary arrest of red cell production resulting in moderate to severe anemia [1-5]. The clinical syndrome is quite uniform with the disease affecting primarily older infants and toddlers, often with a history of a preceding viral illness. There are usually no physical findings other than pallor and a hemic murmur. The blood count reveals a normocytic and normochromic anemia, absent reticulocytes, normal white cell counts, and normal or elevated platelet counts. Bone marrow examination reveals a selective red cell aplasia. Complete hematologic recovery occurs without treatment usually within a month of presentation and recurrences are rare.

The pathogenesis of this fascinating disorder has recently been investigated utilizing clonal assay systems for erythroid stem cell proliferation [6]. Koenig et al. [7] studied four children with TEC and demonstrated a serum or IgG inhibitor of erythroid colony growth in all patients. Dessypris et al. [8] found an IgG inhibitor of erythropoiesis in

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only eight of 12 patients and noted variability of both colony formation at diagnosis and site of inhibitor activity. Freedman and Saunders [9] recently investigated four patients with TEC and concluded that there are at least four mechanisms of erythroid suppression: (1) IgG inhibition, (2) IgM inhibition, (3) cell-mediated inhibition, and (4) nonimmune inhibition. Thus, although the clinical picture is quite typical, the stem cell culture studies are not.

To further document in vitro erythropoiesis in patients with TEC, we studied seven patients with the syndrome utilizing the plasma clot assay system. We assessed (1) the proliferation of erythroid colonies at the time of diagnosis and recovery, (2) the effect of adding the patients' sera to their own bone marrow culture, and (3) the effect of the patients' diagnosis and recovery sera on colony formation from normal control marrow. Our results indicate the presence of a transient serum inhibitor directed against erythroid stem cells in some TEC patients, but in a distinct cohort of patients reduced erythroid proliferation was seen without a demonstrable serum inhibitor. In one patient studied, this diminished erythroid proliferative capacity returned to normal following recovery. These studies document variable in vitro erythropoietic expression in patients with TEC and support the hypothesis of a heterogeneous pathogenesis.

MATERIALS AND METHODS

Patients

The clinical characteristics of the seven patients studied are outlined as follows: There were six boys and one girl ranging in age from $1\frac{1}{12}$ to $3\frac{1}{12}$ years with an average age of $2\frac{1}{2}$ years. Three of the seven patients (patients 1, 2, and 7) had a history of a viral illness in the prior one to two months. Patient 4 was developmentally delayed, had a seizure disorder, and was taking phenytoin and phenobarbital at the time of presentation. Of note is the fact that $2\frac{1}{2}$ years after the diagnosis of TEC was made in patient 4, he presented with hypomegakaryocytic thrombocytopenia and has progressive marrow aplasia. All other patients had received no medication and had an unremarkable past medical history. The average hemoglobin was 6.4 g/dl with a range of 4.8–8.3 g/dl. The MCV was normal for age, and reticulocytes were absent in all cases. White blood counts were within the normal range for all patients, although patient 3 was found to have neutropenia with an absolute granulocyte count of 700. Platelet counts were normal in three and elevated in four. The Coombs test was negative in all patients. Hb F was slightly elevated in two patients (normal <2 percent) and normal in three. Each bone marrow aspirate revealed isolated erythroid hypoplasia with a myeloid-to-erythroid ratio ≥6:1 (normal 2–4:1). Other than red cell transfusions, no treatment was given. Recovery (defined as the first documented reticulocyte count greater than 2 percent) occurred within three weeks for all patients with an average of $1\frac{1}{2}$ weeks. There was no obvious correlation between the time to recovery and the degree of anemia, marrow erythroid hypoplasia, or percentage of Hb F. There have been no recurrences of TEC.

Laboratory Methods

Bone marrow cells were obtained by aspiration from the posterior iliac crest in the patients and 16 normal controls. Bone marrow at diagnosis was obtained from patients 1–4 and at recovery from patient 3. Serum at diagnosis and recovery was obtained from all patients, although insufficient serum was present to complete all allogeneic
studies in patients 1 and 2. Informed consent was obtained from both patients and controls. The plasma clot culture technique was used for cloning erythroid colonies as described by Tepperman et al. [10] except that alpha medium (α-MEM) was substituted for NCTC 109. Dispersed bone marrow cells were layered over Ficoll-Hypaque and centrifuged at 1,500 rpm for 30 minutes at 4°C. The mononuclear cell layer was removed and the cells were washed in supplemented α-MEM + 2 percent fetal calf serum. Bone marrow cells at a final concentration of 6 × 10^5/1.1 ml were cultured in quadruplicate in the presence of 2 IU of Step III sheep erythropoietin. In studies of humoral inhibitors, serum was added to the culture at a final concentration (v/v) of 10 percent. Cultures were maintained in a humidified atmosphere of 5 percent CO₂ at 37°C. At days 6 and 12, clots were removed and transferred to glass slides, fixed in glutaraldehyde, and stained with benzidine and hematoxylin. Erythroid colonies of 8 to 49 benzidine-positive cells appearing on day 6 were counted as CFU-E-derived colonies. Colonies of greater than 50 cells or clusters of three or more CFU-E-derived colonies appearing on day 12 were counted as BFU-E-derived colonies.

Statistical analysis was performed with the Wilcoxon non-paired rank sum test.

RESULTS

Colony Formation at Diagnosis

Figure 1 shows the number of CFU-E-derived colonies from four patients' bone marrow cells obtained at diagnosis. In our laboratory, normal donor marrow cells will produce 58 ± 6 colonies/6 × 10⁴ cells when cultured with 2 IU of erythropoietin. Patient 1's marrow produced 159 ± 15 colonies/6 × 10⁴ cells, over 2½ times the normal number of colonies. Marrow cultures from patients 2 and 3, on the other hand, formed significantly fewer CFU-Es than controls, while no CFU-E-derived colonies formed in cultures of marrow from patient 4. Patient 1 had 1.25 ± 0.5 BFU-E-derived colonies/6 × 10⁴ cells at diagnosis while patients 2–4 had none. In the presence of 2 IU of erythropoietin, BFU-E proliferation in normals in our laboratory varies from 0–7 BFU-E/6 × 10⁴ cells, with an average of 2.5 ± 1.0.

Effect of Serum Obtained at Diagnosis on Autologous Colony Formation

Figure 2 shows the effect of the patient's diagnosis serum and normal control serum on proliferation of CFU-E-derived colonies by the patient's own (autologous) marrow cells. While control serum had no significant effect, serum from patient 1 completely
inhibited colony formation. Serum from patients 2 and 3 had no effect on colony growth relative to control serum. Addition of control serum to patient 4's culture resulted in the appearance of a few colonies, while the patient's own serum increased colony formation eighteenfold. This latter pattern of stimulation has been observed in sera of patients with very high serum erythropoietin levels [11].

With the addition of 10 percent control serum to patient 1's marrow culture, two BFU-E-derived colonies were formed, while none were present with the addition of the patient's serum. Patient 2 had two BFU-E colonies with control serum and one with patient serum. Patients 3 and 4 demonstrated no BFU-E formation with the addition of control or patient serum.

**Effect of Serum Obtained at Diagnosis on Allogeneic Colony Formation**

The effect of diagnosis serum from six of the seven patients on CFU-E colony proliferation by hematologically normal controls is represented in Fig. 3. Patient 1's serum completely inhibited colony formation, as observed in the autologous system (see Fig. 2). Serum from patient 3 produced 17 percent inhibition (patient, 35 ± 2 colonies/6 x 10^4 cells; control, 42 ± 2) and serum from patient 4 produced 17 percent stimulation (patient, 136 ± 16 colonies/6 x 10^4 cells; control, 116 ± 2), respectively, when compared to controls. Differences of this magnitude are within the error of the assay system and are not statistically different at the p < .05 level. These results confirm the lack of inhibition found in the autologous system. Sera from patients 5, 6, and 7 inhibited colony formation by 58, 81, and 60 percent, respectively.

![FIG. 2. Effect of diagnosis serum on autologous CFU-E-derived colony formation. Bone marrow was obtained at diagnosis. Open bars represent the number of colonies formed in the presence of 10 percent control serum; shaded bars represent the number of colonies formed in the presence of 10 percent patient serum at diagnosis. (Result is mean of quadruplicate determination with brackets representing two standard errors of the mean.)](image)

![FIG. 3. Effect of diagnosis serum on allogeneic CFU-E-derived colony formation. The bars represent inhibition or stimulation of colony formation and are expressed as a percentage of control. A shaded bar represents a statistically significant effect.](image)
The effect of diagnosis serum on allogeneic BFU-E proliferation could be assessed in patients 5 and 6. There was insufficient BFU-E growth for similar assessment in the other patients. With the addition of patient 5’s serum to the culture, only $2 \pm 1$ BFU-E were formed compared to $8 \pm 1$ with the addition of control serum. In another experiment, addition of serum from patient 6 completely suppressed BFU-E growth, while with 10 percent control serum $29 \pm 2$ BFU-Es were counted.

**Effect of Serum Obtained After Recovery on Allogeneic Colony Formation**

A comparison of the effect on allogeneic colony proliferation of recovery serum versus diagnosis serum from the four patients with serum inhibitors is shown in Fig. 4. Addition of recovery serum from patient 1 (obtained three months after diagnosis) had no significant effect on CFU-E proliferation when compared to control serum. While only 17 colonies were formed in the presence of diagnosis serum from patient 5, double the number was evident with recovery serum (obtained 3½ weeks after diagnosis), not statistically different than control serum ($p = 0.486$). Early recovery serum from patient 6 (obtained 11 days after diagnosis) had a minimal effect on colony proliferation compared to control serum ($p = 0.056$) and a striking fourfold augmentation compared to diagnosis serum. Recovery serum from patient 7 (obtained two months after diagnosis) had a minimal stimulatory effect when compared to control serum and supported over three times the number of colonies found in cultures containing diagnosis serum.

The effect of recovery serum on BFU-E colony formation in an allogeneic system was assessed in three patients. In patient 1, recovery serum had no effect on colony formation when compared to control serum ($7 \pm 1$ vs. $9 \pm 2$ BFU-Es). Similar results were obtained utilizing patient 5’s recovery serum ($6 \pm 1$ vs. $8 \pm 1$ BFU-Es). With the early recovery serum from patient 6, however, only two BFU-E-derived colonies were enumerated, compared to 29 colonies with control serum.

**Colony Formation After Recovery**

One of three patients without evidence of an inhibitor had a bone marrow aspirate performed after complete hematologic recovery. Six weeks after diagnosis, patient 3 had a normal hemoglobin (11.5 g/dl), a bone marrow with normal erythroid elements, and a normal M:E ratio of 2.5:1. Figure 5 shows CFU-E-derived colony formation of this patient’s recovery marrow compared to colony growth at diagnosis and to growth
of control marrow cells. While at diagnosis there were only 18 CFU-E-derived colonies/6 $\times$ $10^4$ cells, at recovery CFU-E proliferation increased sevenfold to 128 colonies, twice that of controls. BFU-E-derived colony formation revealed a similar increase in proliferative capacity, with 19 colonies counted in the culture of recovery marrow. This is a dramatic increase when compared to the absent growth at diagnosis and considerably higher than our normal range for BFU-E numbers.

**DISCUSSION**

Previous studies of patients with TEC utilizing clonal stem cell assays for erythroid stem cell proliferation have increased our understanding of the mechanism of this disorder, yet there are inconsistencies in the reported results. Koenig et al. [7] described the first in vitro studies in patients with TEC. Significant inhibition of allogeneic CFU-E-derived colony formation occurred with the addition of serum of IgG from all four patients studied. There was variability, however, of the effect on BFU-E growth. In the only patient whose own marrow was studied, normal erythroid colony growth was observed but was abolished by addition of her serum. They concluded that an immune mechanism suppressing erythroid proliferation is the most likely cause of the anemia in TEC.

Dessypris et al. [8] studied 12 patients with TEC. Erythroid stem cell proliferation of the patient's own marrow was studied in nine patients. Five of these patients had normal colony numbers, but four had decreased numbers. In the five patients with normal in vitro erythropoiesis, only three could be shown to have serum and IgG-mediated suppression of colony formation. Of the four patients with diminished erythroid colonies at diagnosis, no inhibition could be demonstrated in an autologous system, while IgG from two of the four patients caused suppression of erythropoiesis in an allogeneic system. IgG from two additional patients studied in an allogeneic system suppressed BFU-E growth without affecting CFU-E growth. These studies first raised the question of mechanisms other than immune suppression as the cause of the anemia in TEC.

Freedman and Saunders [9] reported studies on four children with TEC. Three of the four had normal erythroid colony formation at diagnosis, while one had low numbers of CFU-Es and BFU-Es. Of the patients with normal colony formation at diagnosis, one was found to have a serum and IgG inhibitor, the second had a serum and IgM inhibitor with no evidence of IgG inhibition, and in the third no inhibitor was demonstrated. The patient with diminished erythroid capacity at diagnosis had evidence of cell-mediated inhibition in co-culture studies.
In the present report, variability of in vitro erythropoiesis in seven patients with TEC is again noted. Only one of four patients studied had normal colony formation at diagnosis. This patient had clear evidence of serum inhibition of both autologous and allogeneic CFU-E-derived colony formation. In the three patients with diminished in vitro erythroid potential at diagnosis, no serum inhibition of colony formation could be demonstrated. Furthermore, in one of these patients studied after complete recovery, erythroid proliferative capacity returned to supernormal levels. Three additional patients whose serum only was studied had evidence of inhibition of erythropoiesis. Recovery sera from patients with inhibitors showed no evidence of suppression of colony formation, as noted in the patients studied by the previous authors. BFU-E data from these studies were more difficult to interpret because of low colony numbers, but in general coincided with the CFU-E results.

It can be concluded from the foregoing studies that the anemia of TEC in many cases is mediated by a serum inhibitor directed at the erythroid-committed stem cell. In most instances, this inhibitor is an IgG immunoglobulin, but, rarely, an IgM immunoglobulin has been implicated. This type of mechanism is not without precedent, having been demonstrated in both a child [12] and adults [13] with aregenerative anemias. Although the stimulus for development of this autoantibody is unknown, the many similarities of TEC with childhood idiopathic thrombocytopenia purpura, felt to be triggered by a prior viral infection, would suggest a viral etiology.

Evidence for serum inhibition of erythroid colony formation in patients with congenital hemolytic anemia in the midst of an aplastic crisis has been reported by Mortimer et al. [14]. Inhibition in these patients, however, appears to be due to a serum parvovirus-like virus (SPLV) and not by an immune mechanism [15]. Sera from our patients with TEC were tested for SPLV antigen (kindly performed by Dr. Neal Young) and found to be negative, as have previous studies of TEC sera [15]. Thus, direct viral suppression by SPLV does not appear to be an explanation for the serum inhibition seen in patients with TEC.

It is also clear from the studies of patients with TEC that not all will have the typical in vitro findings which are seen in those with inhibitors. Most of these "atypical" patients have decreased erythroid proliferation at diagnosis without demonstrable serum inhibition of colony formation in an autologous system. Since cell-mediated suppression of erythropoiesis was found in one patient with TEC as well as having been reported in patients with other types of anemia [16, 17], this could be an explanation for the findings. Nonimmune mechanisms directly toxic to erythroid progenitors and limiting their normal proliferation or differentiation could also explain the results. Finally, in some instances the assay system may be too insensitive to demonstrate a weak serum inhibitor.

It is of interest that although in vitro erythropoiesis is variable, the clinical syndrome is usually quite predictable. There are, however, some exceptions. The patient studied by Freedman and Saunders with no evidence of serum or cell-mediated suppression of colony formation was atypical because of her older age at presentation (ten years), the presence of hepatosplenomegaly, and a positive antinuclear factor. Patient 4 in the present study had decreased erythroid colonies at diagnosis with no demonstrable serum inhibitor and, although he had the usual course for TEC, subsequently developed marrow failure. Another patient described by Freedman [18] presented with the typical findings of TEC, yet had a prolonged course with a recurrent episode of anemia and reticulocytopenia. This patient had normal erythropoiesis at diagnosis with evidence of a serum inhibitor, which was shown to be an IgM immunoglobulin and not
the more frequently found IgG. The clinical course of these patients as well as the variable in vitro erythropoiesis of this disorder support the hypothesis of a heterogeneous pathogenesis. With further refinements of in vitro technique, increased understanding of both the immune and possibly the nonimmune mechanisms of the disease, and further correlation of the clinical course with the laboratory data, a clearer picture of TEC may be possible.

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