Mode of Action of the IgG Inhibitor of Erythropoiesis in Transient Erythroblastopenia of Childhood

By Emmanuel N. Dessypris, Sanford B. Krantz, James S. Roloff, and John N. Lukens

Twelve cases of transient erythroblastopenia of childhood (TEC) have been studied to evaluate their marrow cell erythropoiesis in vitro and the effect on it of their serum or IgG. The number of colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) in the bone marrow of nine cases was extremely variable and did not allow any conclusion regarding the pathogenesis of this anemia. An IgG inhibitor of growth of erythroid colonies or bursts was detected in 8/12 cases. This IgG inhibitor had no effect on the growth of granulocyte-macrophage colonies. Further studies on its mode of action indicated that the IgG did not have antierythropoietin antibody properties and did not affect the mature erythroblasts, as shown by a lack of inhibition of their response to erythropoietin and by the lack of a cytotoxic effect on 59Fe-labeled erythroblasts.

In four cases, preincubation studies demonstrated a direct effect of the IgG on the CFU-E, which was complement-mediated in three cases and complement-independent in one case. In two other cases, the IgG suppressed the growth of normal BFU-E only without affecting the growth of CFU-E. The IgG inhibitor was no longer present after the erythroblastopenia had remitted. These studies demonstrate that in the majority of cases of TEC, an IgG suppressor of erythropoiesis in vitro is present. Its mode of action is heterogeneous regarding its requirement for complement. Its target cells are the earlier or later erythroid progenitors, BFU-E or CFU-E, but not the differentiated erythroblasts.

Transient erythroblastopenia of childhood (TEC) is a syndrome characterized by disappearance of the erythroblasts from the bone marrow followed by reticulocytopenia in the blood and development of severe normochromic normocytic anemia. It affects hematologically normal children, who generally are older than 6 mo of age, and remits spontaneously and completely. TEC is differentiated from congenital hypoplastic anemia (Diamond-Blackfan syndrome) by the absence of anemia during the first 6 mo of life, the normal concentration of HbF and i, red cell antigens, as well as by a decline of age-dependent enzymes in circulating red cells, all indicative of a complete acute arrest of erythropoiesis in the bone marrow. Furthermore, its acute and self-limited course is in contrast to the chronic, relapsing, and steroid-dependent nature of congenital hypoplastic anemia.

A previous study by Koenig et al. has demonstrated the presence of an IgG inhibitor of normal marrow erythropoiesis in vitro in the serum of four affected children. These investigators suggested that the anemia of TEC is due to transient immune suppression of erythroid colony development. Since erythropoiesis has three basic components, erythropoietin, erythropoietin-responsive cells, and erythroblasts that arise from the action of erythropoietin on the erythropoietin-responsive cells, an immune factor could reduce erythropoiesis by acting on any one of these components. The observation of reduced erythroid colony formation in vitro in the presence of patient serum or IgG does not allow any conclusions as to which of the above three basic components of erythropoiesis an inhibitor is directed. In the present study an attempt was made to characterize the target cell(s) of the IgG inhibitor of erythropoiesis in TEC. In 8 of the 12 studied cases, an IgG inhibitor of erythropoiesis was detected. This IgG had no antierythropoietin antibody properties and did not affect the mature erythroblasts as indicated by the normal response of the latter to the hormone in the presence of the IgG. In four cases, the IgG was directed against the CFU-E themselves, as opposed to the erythroid cells that develop from the CFU-E. In three of them the inhibition was complement-mediated and in the remaining case, complement-independent. In two cases, the IgG suppressed the growth of BFU-E only, leaving the CFU-E growth unaffected. This indicated that in these cases the arrest of erythropoiesis occurred at a cell level earlier than the CFU-E. In the remaining two patients, inclusion of IgG in the culture medium depressed the growth of both CFU-E and BFU-E, but a specific direct effect on the CFU-E or BFU-E could not be demonstrated. These studies demonstrate that in the majority of cases of TEC, an IgG inhibitor of erythropoiesis in vitro can be identified and that its mode of action...
action is heterogeneous in regard to its target cell and its requirement for complement.

MATERIALS AND METHODS

Human Subjects

Twelve children with TEC were included in this study. The criteria for the diagnosis of this syndrome were: (1) normochromic normocytic anemia with reticulocytopenia; (2) severe bone marrow erythroid hypoplasia or aplasia with normal numbers of granulocytic and megakaryocytic cells; (3) spontaneous and complete recovery. All studies patients fulfilled the above criteria except patients 2 and 11 who showed an increased number of early proerythroblasts in the bone marrow with a complete lack of more mature erythroblasts, a finding previously reported in TEC.8 The proerythroblasts in the bone marrow with a complete lack of more mature erythroblasts, a finding previously reported in TEC.8 The marrow aspiration was performed and blood was collected at the time of diagnosis before any transfusion of red cells was given. None of the studied patients had a history of previous transfusion with red cells or other blood products. In most cases blood was collected also following the spontaneous remission of the syndrome.

Normal blood and marrow samples were obtained from normal healthy adult volunteers after informed consent. All serum samples from patients and normal individuals were stored at –20°C until they were used. All the studies were approved by the Vanderbilt Committee for the Protection of Human Subjects.

Marrow Cell Cultures

CFU-E and BFU-E Assay

Aspirated marrow cells were placed in a sterile polystyrene tube containing heparin (5 U/ml) and were passed through a 10-ml pipette 10 times to obtain adequate dispersion. They were then layered over an equal volume of a mixture of Ficoll-Hypaque in water (d = 1.077) before being centrifuged at 400 g for 20 min at room temperature. The cells at the interface between the plasma and Ficoll-Hypaque were collected, washed twice with 10 ml of the plasma clot system described by McLeod et al.9 and modified by Clarke and Housman.9 For the quantitation of CFU-E and BFU-E, 4–6 clots were counted and the results were expressed as mean ± standard error of the mean (SEM). The effect of patient serum on the growth of CFU-E and BFU-E was assessed by replacing 0.05–0.1 ml of normal human serum by an equal volume of patient’s serum (final concentration 5—10%) after heat-treating it at 56°C for 30 min. IgG was added as part of the a-MEM, providing a final concentration of 0.6–1.2 mg/ml of culture medium. An IgG was considered inhibitory when it suppressed the growth of erythroid progenitors beyond 42% of the normal pooled AB serum IgG. This definition was derived from 8 experiments in which the latter was compared to the effect of IgG from 8 different normal subjects upon their own marrow cells in vitro. From these experiments, the range (mean ± 2 standard deviations) of the effect of autologous IgG on the growth of normal erythroid progenitors was found to be ± 42% of the control pooled AB serum IgG.

Pretreatment of Bone Marrow Cells With IgG and Complement

Two-tenths milliliter aliquots of the above light density bone marrow cells were transferred to 12 × 75 mm polystyrene plastic tubes, containing 0.1 ml α-MEM or 0.1 ml solution of IgG in α-MEM (0.6–1.2 mg/ml) and 0.3 ml of fresh frozen AB human serum was transferred to 0.7 ml of culture medium consisting of 0.3 ml human serum pooled from 20 normal donors of blood type AB and heat treated at 56°C for 30 min; 0.1 ml 10% deionized bovine serum albumin, 0.25 ml α-MEM; 0.03 ml of a fibrinogen solution (40 mg/ml), 1 ml of which was dialyzed in 300 ml Hanks’ balanced salt solution (HBSS, Gibco, Grant Island, N.Y.) for 3 hr; and 0.02 ml of erythropoietin (sheep plasma erythropoietin; Step III, Connaught Laboratories, Ontario, Canada) providing a final concentration of 1 U/ml for the growth of CFU-E and 2 U/ml for the growth of BFU-E. Clotting was induced by the addition of 0.1 ml bovine citrated plasma and 0.1 ml of a solution of bovine thrombin containing 1 U/ml. Following the addition of thrombin and before clotting occurred, 0.1 ml of the medium was transferred to 0.25-ml wells in a disposable microtiter tray (Cooke, Alexandria, Va.). The cells were incubated at 37°C, in a 5% CO2 atmosphere with high humidity for 7 or 15 days, when the plasma clots were fixed and stained with benzidine-hematoxylin as described by McLeod et al.9 CFU-E were enumerated on day 7 by counting all benzidine-positive colonies consisting of 8 or more cells. BFU-E were enumerated on day 15 according to the criteria described by Clarke and Housman.9 For the quantitation of CFU-E and BFU-E, 4–6 clots were counted and the results were expressed as mean ± standard error of the mean (SEM). The effect of patient serum on the growth of CFU-E and BFU-E was assessed by replacing 0.05–0.1 ml of normal human serum by an equal volume of patient’s serum (final concentration 5—10%) after heat-treating it at 56°C for 30 min. IgG was added as part of the α-MEM, providing a final concentration of 0.6–1.2 mg/ml of culture medium. An IgG was considered inhibitory when it suppressed the growth of erythroid progenitors beyond 42% of the normal pooled AB serum IgG. This definition was derived from 8 experiments in which the latter was compared to the effect of IgG from 8 different normal subjects upon their own marrow cells in vitro. From these experiments, the range (mean ± 2 standard deviations) of the effect of autologous IgG on the growth of normal erythroid progenitors was found to be ± 42% of the control pooled AB serum IgG.

Table 1. Hematologic Values of the 12 Patients With TEC at the Time of Diagnosis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (mo)</th>
<th>PCV* (%)</th>
<th>MCV† (fl)</th>
<th>Reticulocytes (%)</th>
<th>WBC‡ (× 10³/liter)</th>
<th>Platelets (× 10³/liter)</th>
<th>Follow-up Period After Remission (mo)</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>23</td>
<td>23.4</td>
<td>81</td>
<td>0</td>
<td>8.4</td>
<td>968</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>19</td>
<td>22.6</td>
<td>78</td>
<td>0.1</td>
<td>7.0</td>
<td>570</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>90</td>
<td>16.5</td>
<td>82</td>
<td>0</td>
<td>4.3</td>
<td>300</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>8</td>
<td>15.0</td>
<td>76</td>
<td>0.1</td>
<td>6.7</td>
<td>501</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>24</td>
<td>17.7</td>
<td>79</td>
<td>0</td>
<td>13.3</td>
<td>592</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>23</td>
<td>18.5</td>
<td>85</td>
<td>0</td>
<td>6.9</td>
<td>347</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>7</td>
<td>19.2</td>
<td>78</td>
<td>0</td>
<td>6.0</td>
<td>528</td>
<td>6</td>
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<tr>
<td>8</td>
<td>F</td>
<td>27</td>
<td>14.8</td>
<td>79</td>
<td>0</td>
<td>5.5</td>
<td>347</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>20</td>
<td>13.9</td>
<td>73</td>
<td>0.1</td>
<td>12.8</td>
<td>950</td>
<td>9</td>
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<tr>
<td>10</td>
<td>M</td>
<td>19</td>
<td>17.1</td>
<td>76</td>
<td>0.6</td>
<td>5.2</td>
<td>267</td>
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<td>M</td>
<td>16</td>
<td>19.0</td>
<td>86</td>
<td>0.3</td>
<td>7.2</td>
<td>406</td>
<td>4</td>
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<tr>
<td>12</td>
<td>M</td>
<td>24</td>
<td>13.7</td>
<td>77</td>
<td>0.6</td>
<td>6.9</td>
<td>537</td>
<td>4</td>
</tr>
</tbody>
</table>

*Packed cell volume.
†Mean corpuscular volume.
‡White blood cells.
serum as a source of complement. In some experiments the comple-
ment present in the serum was inactivated by preheating the serum to
56°C for 30 min, or complement was inhibited by the addition of
0.01 ml of disodium ethylenediaminetetraacetate (EDTA) in
normal saline providing a final concentration of 3 mM EDTA. The
cell suspensions were gassed with CO₂, capped, and incubated in a
water bath for 1 hr at 37°C. At the end of the incubation 3 ml of
α-MEM were added to each tube, and the cells were centrifuged at
100 g for 5 min. The supernatants were discarded and the cell pellets
were washed once again with 3 ml of α-MEM, before being finally
resuspended in 0.2 ml of α-MEM. One-tenth milliliter of the cell
suspending was transferred to the above culture medium and was
assayed for CFU-E and BFU-E. In these experiments the culture
medium without the cells, as well as the bovine citrated plasma,
were heat treated at 56°C for 30 min.

Heme Synthesis In Vitro by Bone Marrow
Erythroblasts

Short-term marrow cell liquid cultures were performed as
previously described. Aspirated bone marrow cells were collected
and dispersed as described above. They were then washed twice
with 10 ml of HBSS and suspended at a concentration of 5 × 10⁶
cells/ml in NCTC-109. One-tenth milliliter of this bone marrow cell suspension was transferred to 35 × 20 mm tissue culture dishes containing
0.9 ml of culture medium, which consisted of 30% NCTC-109
(Microbiological Associates, Bethesda, MD), 30% α-MEM, 20%
human normal serum, 0.8% methylcellulose (Fisher Scientific, Fairtown, N.J.), 2%
conditioned medium. Serum as a source of complement. In some experiments the comple-
ment present in the serum was inactivated by preheating the serum to
56°C for 30 min, or complement was inhibited by the addition of
0.01 ml of disodium ethylenediaminetetraacetate (EDTA) in
normal saline providing a final concentration of 3 mM EDTA. The
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water bath for 1 hr at 37°C. At the end of the incubation 3 ml of
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100 g for 5 min. The supernatants were discarded and the cell pellets
were washed once again with 3 ml of α-MEM, before being finally
resuspended in 0.2 ml of α-MEM. One-tenth milliliter of the cell
suspending was transferred to the above culture medium and was
assayed for CFU-E and BFU-E. In these experiments the culture
medium without the cells, as well as the bovine citrated plasma,
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0.9 ml of culture medium, which consisted of 30% NCTC-109
(Microbiological Associates, Bethesda, MD), 30% α-MEM, 20%
normal human heat-treated AB serum, and 20% newborn calf serum. IgG was added as part of the α-MEM at a final concentra-
tion of 0.6–1.2 mg/ml of culture medium. Sheep plasma erythro-
poetin Step III was added to 1 ml of the culture medium to provide
0.6–1.2 mg/ml to be tested or α-MEM was added. Following incubation
overnight at 37°C, the tubes were centrifuged at 1000 g
for 5 min. The supernatants were collected and both pellets and
supernatants were counted in an automatic gamma well-type scintil-
lation counter. The results were expressed as a release index (RI)

\[
RI = \frac{\text{Radioactivity of the supernatant} \times 10^5}{\text{Total radioactivity}}
\]

It has been previously demonstrated that the ⁵⁷Fe radioactivity in
the supernatant represents loss of radioactive hemoglobin from
the erythroblasts and is an index of erythroblast cytotoxicity.

Assay of Colony-Forming Units-Granulocyte,
Macrophage (CFU-GM)

Cells from the light density fraction of the bone marrow were
cultured using the methylcellulose method with colony-stimulating activity (CSA) derived from peripheral human blood leukocyte conditioned medium as described by Iscove et al.²¹ The culture medium consisted of α-MEM containing 20% heat-treated fetal calf serum, 0.8% methylcellulose (Fisher Scientific, Fairtown, N.J.), 2%
deionized bovine serum albumin, and 20% conditioned medium.²¹
IgG was added as part of the α-MEM at a final concentration of
0.6–1.2 mg/ml. After addition of 2 × 10⁵ cells/ml of culture medium, 1 ml of the mixture was transferred to 35 × 20 mm tissue culture
dishes and incubated at 37°C, 5% CO₂, and high humidity for
10 days. At the end of the incubation period, colonies containing
more than 50 cells were counted using an inverted microscope.

Isolation of Serum IgG

Serum immunoglobulins were purified by ammonium sulfate precipitation²² and DEAE cellulose chromatography.²³ It has
previously been shown that these preparations were free of impuri-
ties demonstrable by immunoelectrophoresis.²⁴ The IgG solutions
were sterilized by filtration through a 0.45 µm pore size Millipore
filter and were stored at -20°C. Protein concentrations were
measured spectrophotometrically by the absorbance at a wave
length of 280 nm. Normal human serum Ig was isolated from the
pooled sera of 20 type AB donors.

RESULTS

CFU-E and BFU-E Growth From Patients’ Marrows

The proliferative capacity of the erythroid progenitors, CFU-E and BFU-E, was assessed in 9 cases of TEC (Table 2). In 5/9 cases, a normal number of CFU-E/10⁶ plated nucleated bone marrow cells was observed and in the remaining 4 cases, a subnormal number of CFU-E developed into erythroid colonies in
Table 2. Erythroid Colony and Burst Formation In Vitro From the Marrow Cells of 9 Patients With TEC

<table>
<thead>
<tr>
<th>Patient</th>
<th>CFU-E/10^5 Nucleated Cells*</th>
<th>BFU-E/10^5 Nucleated Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64 ± 9.0</td>
<td>21 ± 5.0</td>
</tr>
<tr>
<td>2</td>
<td>47 ± 11.0</td>
<td>34 ± 7.0</td>
</tr>
<tr>
<td>3</td>
<td>132 ± 22.0</td>
<td>52 ± 12.0</td>
</tr>
<tr>
<td>4</td>
<td>12 ± 4.0</td>
<td>11 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>6 ± 0.4</td>
<td>3 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>4 ± 0.5</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>74 ± 8.0</td>
<td>50 ± 8.0</td>
</tr>
<tr>
<td>11</td>
<td>135 ± 16.0</td>
<td>29 ± 2.0</td>
</tr>
<tr>
<td>12</td>
<td>5 ± 0.5</td>
<td>1 ± 0.4</td>
</tr>
</tbody>
</table>

Normal (n = 25) 236 ± 42 (range 57-554) 35 ± 8 (range 8-98)

*Mean ± SEM of quadruplicates.

Similarly, in 6/9 cases, the growth in vitro of the patients' bone marrow BFU-E was found to be normal and in 3/9 cases subnormal. In general, the growth of the erythroid progenitors of the marrow of patients with TEC was found to be quite variable, having no correlation with any clinical parameter or the presence or absence of an IgG inhibitor of erythropoiesis in the patients' sera.

Effect of Patients' Sera on the Growth In Vitro of Autologous Marrow CFU-E and BFU-E

When 10% of normal human sera used in the culture medium was replaced by an equal volume of patients' sera a suppression of autologous CFU-E and BFU-E growth was observed in 3/5 evaluable cases (patients 1, 2, and 3) (Fig. 1). In four other cases (patients 4, 5, 6, and 12) the growth of the patients' marrow in vitro was so poor that no conclusion could be drawn regarding the presence of a serum inhibitor of the erythroid growth.

Effect of Patients' IgG on the Growth of Autologous and Normal Marrow CFU-E and BFU-E

Addition of 0.6–1.2 mg of purified patients' IgG/ml of culture medium resulted in significant depression of autologous CFU-E and BFU-E growth in vitro (Fig. 2), similar in magnitude to that observed following the addition of 10% autologous serum (patients 1, 2, and

![Fig. 1. Effect of 10% patients' sera (□) or normal pooled serum (▲) on the growth of autologous CFU-E and BFU-E. Mean ± SEM of quadruplicates.](image-url)
In Fig. 2, the effect of patients' IgG (□) or normal pooled IgG (■) on the growth of autologous CFU-E and BFU-E in vitro is shown. Mean ± SEM of quadruplicates.

3). Purified IgG from the sera of 12 children with TEC, when added to the culture medium at a concentration of 0.6–1.2 mg/ml, suppressed the growth of normal marrow CFU-E in 4/12 cases and the growth of normal marrow BFU-E in 6/12 cases (Fig. 3). In two cases (patients 9 and 10), the serum IgG exerted its inhibitory effect on the growth of CFU-E only, leaving the growth of BFU-E unaffected. The inhibitory effect of the patients' IgG was parallel to the disease activity and was no longer present in the sera of patients in remission. As shown in Fig. 4, as the disease progressed towards remission, the inhibitory effect of the serum IgG decreased and disappeared completely in the serum drawn in remission.

**Pretreatment of Marrow Cells With IgG and Complement**

Preincubation of marrow cells with α-MEM, alone or with human AB serum, heat-treated AB serum, or human AB serum plus EDTA produced no differential effect on the growth of the CFU-E (Fig. 5). Similarly, preincubation of the marrow cells with normal IgG, alone or with the above fresh or heat-treated human serum, did not affect CFU-E development. In one case (patient 3), incubation of the bone marrow cells with the patient IgG alone depressed the growth of CFU-E, indicating that the IgG was acting directly on the CFU-E in a complement-independent way. The addition of fresh human serum did not alter this effect. In three other cases (patients 1, 2, and 8), preincubation of the marrow cells with the patient's IgG and fresh human serum markedly depressed the growth of CFU-E. This effect was abolished completely by heat treating the serum or inhibiting complement by the addition of EDTA, indicating that in these three cases the serum IgG was acting directly on the CFU-E through a complement-dependent mechanism (Fig. 5). This effect of IgG was no longer present in the serum drawn after remission of the TEC (data not shown). Using similar experiments, no direct effect of the IgG on the CFU-E was detected in the remaining eight cases. Similar experiments designed to detect any possible direct effect of the patient serum IgG on the BFU-E were not successful mainly because of a nonspecific depression of the BFU-E growth after incubation of the marrow cells with fresh human serum.

**Effect of Patients' Sera IgG on Heme Synthesis by Normal Bone Marrow Cells In Vitro**

As shown in Fig. 6, normal bone marrow cells incorporated the same amount of ^59^Fe into heme in the presence of the patients' IgG as compared to normal IgG and responded to erythropoietin normally by a twofold increase in heme ^59^Fe.

**Cytotoxic Effect of Serum IgG on Bone Marrow Erythroblasts**

When the radioactive erythroblasts were exposed to patient serum IgG and human AB serum as a source of complement, the release of ^59^Fe in the supernatant following 20 hr of incubation at 37°C was equal to the release of ^59^Fe by normal human serum IgG indicative of an absence of direct cytotoxicity of the tested serum IgG on the mature erythroid cells in the marrow (Table 3). In contrast, serum IgG from an adult patient with chronic pure red cell aplasia (PRCA) and an anterythroblast antibody produced an almost twofold increase of the release of ^59^Fe compared to normal IgG.

**Effect of Patients' IgG on Development of CFU-GM**

When patient's serum IgG was added to the culture medium at a final concentration of 0.6–1.2 mg/ml, the growth of normal bone marrow CFU-GM remained...
unaffected and equal to the growth observed in the presence of normal human IgG (Table 4).

A summary of the results of in vitro findings in each one of the 12 studied cases is presented in Table 5.

DISCUSSION

Previous studies by Koenig et al. demonstrated the presence of a serum IgG inhibitor of erythroid colony formation in four children with TEC and suggested that this syndrome may have an immune pathogenesis. The present study was undertaken in an attempt to explore the extent of these observations and to further characterize the target cell(s) of the IgG inhibitor and its mode(s) of action on erythroid cells.

The simple quantitation of the growth of the erythroid progenitors CFU-E and BFU-E in the marrow of nine children with TEC did not provide any information relevant to the possible pathogenesis of this syndrome. In cases of suppression of erythropoiesis secondary to an environmental factor, such as the case of immune suppression, it is expected that when the cells are freed of their environment and grown in vitro, a normal type of growth may be observed. The variable growth of CFU-E and BFU-E in the nine studied cases could be explained solely by the notorious sensitivity of this culture system to minor changes in the culture conditions. However, such an explanation seems unlikely, since normal control marrow cultures always were performed at the same time. It seems more likely that the erythroid progenitors in the marrow of some cases may be affected to a variable degree by the ongoing process in vivo, which renders them unable to recover and to proliferate normally in vitro. Thus, while depressed erythroid growth in vitro may occur as an expression of a defective progenitor cell in the marrow, it might also arise as a variable expression of progenitor cell change by environmental factors.

The presence of a serum inhibitor of the CFU-E and/or BFU-E growth in vitro was shown in three of six evaluable cases (cases 1, 2, and 3) in an autologous system. It was subsequently found that in these three cases the inhibitory effect of the serum was due to its IgG, which when added to the culture medium suppressed, in equal proportion as the serum, the growth of autologous erythroid progenitors. In two of these three cases the same effect of the serum IgG was observed on the growth of normal CFU-E/BFU-E. It seems that in some cases, an autologous system (serum
Fig. 5. Effect of pretreatment of normal marrow cells with patients' IgG with or without complement on CFU-E growth in vitro. Preincubation with α-MEM or IgG (□): α-MEM or IgG plus fresh human serum as a source of complement (■): α-MEM or IgG plus human serum heat treated at 56°C for 30 min (▲): α-MEM or IgG plus fresh human serum and 3 mM EDTA (●). The results represent the mean ± SEM of quadruplicates from three different experiments.

Fig. 6. Effect of patients' IgG on heme synthesis by normal human bone marrow cells in vitro. The bone marrow cells were cultured with erythropoietin (■) or without (□) for 40 hr before adding 0.3 μCi 59Fe. Cultures were then terminated 20 hr later, and heme was extracted and counted for radioactivity. The results represent the mean ± SEM of quadruplicates.

Table 3. 59Fe Release IndexProduced in Normal Marrow Cells by the Serum IgG From 10 Cases of TEC and 1 Case of Adult Chronic Pure Red Cell Aplasia (PRCA)

<table>
<thead>
<tr>
<th>IgG</th>
<th>59Fe Release Index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.98 ± 0.23</td>
</tr>
<tr>
<td>Normal</td>
<td>7.13 ± 0.14</td>
</tr>
<tr>
<td>Pt. 1</td>
<td>6.87 ± 0.39</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>7.27 ± 0.48</td>
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<tr>
<td>Pt. 3</td>
<td>6.99 ± 0.29</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>6.68 ± 0.48</td>
</tr>
<tr>
<td>Pt. 5</td>
<td>7.21 ± 0.09</td>
</tr>
<tr>
<td>Pt. 6</td>
<td>7.11 ± 0.37</td>
</tr>
<tr>
<td>Pt. 7</td>
<td>7.18 ± 0.48</td>
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<tr>
<td>Pt. 8</td>
<td>6.76 ± 0.46</td>
</tr>
<tr>
<td>Pt. 9</td>
<td>6.38 ± 0.54</td>
</tr>
<tr>
<td>Pt. 10</td>
<td>7.03 ± 0.53</td>
</tr>
<tr>
<td>PRCA</td>
<td>12.26 ± 0.53</td>
</tr>
</tbody>
</table>

*Mean ± SEM of quadruplicates.

Table 4. CFU-GM/2 × 10^6 Normal Nucleated Bone Marrow Cells Grown in the Presence of Patients' Sera IgG or Normal IgG

<table>
<thead>
<tr>
<th>IgG</th>
<th>CFU-GM/2 × 10^6 Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>Normal</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Pt. 1</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>Pt. 5</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>Pt. 6</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Pt. 7</td>
<td>48 ± 8</td>
</tr>
<tr>
<td>Pt. 8</td>
<td>53 ± 1</td>
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<tr>
<td>Pt. 9</td>
<td>57 ± 6</td>
</tr>
<tr>
<td>Pt. 10</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>Pt. 11</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Pt. 12</td>
<td>51 ± 4</td>
</tr>
</tbody>
</table>

*Mean ± SEM of quadruplicates.
IgG and bone marrow from the same patient) might provide a more sensitive system for the detection of immune suppression than normal allogeneic bone marrow. A similar observation was made by Roodman et al.\(^26\) in a case of pancytopenia associated with lymphocyte inhibition of erythroid and myeloid colony growth in vitro. However, the explanation for this effect is not known.

When serum IgG from 12 children with TEC was assayed on normal bone marrow cells, suppression of CFU-E growth was detected in 4/12 cases and of BFU-E growth in 6/12 cases. In 2 cases the patients' sera IgG affected only the BFU-E growth and had no effect on the growth of the CFU-E. Since the growth of the BFU-E in vitro requires the presence of other cells or humoral factors produced by nonerythroid cells,\(^27\)\(^29\) it is difficult to distinguish whether the suppression of the BFU-E growth was a result of a direct effect of the IgG on the BFU-E per se or on another cell required for the BFU-E growth. Nevertheless, in these two patients it is clear that the suppression of erythropoiesis in vitro occurred at the level of the burst-forming cell and not the colony-forming erythroid precursor. The lack of effect of all patients' sera IgG on the growth of CFU-GM in vitro demonstrates its specificity for the erythroid progenitors.

In order to understand further the mode of action of the IgG inhibitor of erythroid colony growth, a series of experiments was performed in which the serum IgG was not incorporated in the culture medium, but the cells were incubated with the IgG before being plated. The preincubation was performed in the presence or absence of complement, and the cells were then washed and cultured in a complement-free medium. These experiments showed that in 4/12 cases pretreatment of the bone marrow cells with IgG, with or without complement, was enough to suppress the CFU-E growth in vitro, demonstrating that the serum IgG was directed against the CFU-E cell in the marrow. Prior studies incorporated the IgG into the cell culture medium and did not delineate whether the target cell was the erythroid progenitor or its derivatives, mature erythroblasts. Further investigation of the role of complement demonstrated that in 3/4 cases the suppression of CFU-E growth following treatment of the CFU-E with patients' IgG required the presence of complement, while in the fourth case the suppression of CFU-E growth was complement independent. The use of normal instead of autologous marrow in these studies became necessary for practical reasons. However, none of the patients studied had previously received blood transfusions; hence the effect of the serum IgG on the CFU-E cannot be attributed to acquired transfusion-related alloantibodies. Moreover, whether normal or autologous marrow was used, the inhibitory effect of the IgG on the growth of erythroid progenitors in vitro was demonstrable only with sera drawn at the time of diagnosis of TEC when the suppression of erythropoiesis in vivo was maximum and not in sera drawn after remission, indicating that the phenomena observed in vitro were related to the disease activity in vivo.

Previous studies in adults with chronic idiopathic pure red cell aplasia have demonstrated the presence in their sera of an IgG inhibitor of marrow cell heme synthesis\(^30\) and erythroid colony growth in vitro,\(^30\) a cytotoxic IgG against their erythroblasts,\(^18\)\(^19\) and an antibody to erythropoietin.\(^31\) Since the observations in this group of children demonstrate the presence of an IgG inhibitor of erythropoiesis in vitro, which is directed against the erythroid progenitor cells, the
question arose whether the same IgG also had an effect on mature erythroblasts or on the action of erythropoietin. A series of experiments were performed in which heme synthesis by normal bone marrow erythroid cells was measured in vitro in the presence of normal or patient's IgG, with and without erythropoietin. Normal bone marrow cells had a twofold increase in heme synthesis with the hormone and synthesized the same amount of heme in the presence of normal or patients' IgG, demonstrating that the patients' IgG did not affect the mature erythroblasts and did not have properties of an anti-erythropoietin antibody. Furthermore, an erythroblast cytotoxicity assay failed to demonstrate any cytotoxic effect of the patients' IgG on normal $^{59}$Fe-labeled erythroblasts. Thus, our investigation indicates that the inhibitory effect of the IgG in these patients with TEC is not extended to the mature erythroid cells in the marrow and that the inhibition of erythropoiesis occurs at an earlier stage of differentiation.

These studies demonstrate the value of multiple techniques to identify and characterize an IgG inhibitor of erythropoiesis. By the use of various methods, an IgG inhibitor of growth of the erythroid progenitors in vitro can be demonstrated in the majority of patients with TEC. There remain, however, a number of cases in which no inhibition by the patients' sera IgG can be identified. In these cases, either the methods used were not sensitive enough to allow detection of an IgG inhibitor or another mechanism of suppression of erythropoiesis may have been present. It seems that TEC may not be pathogenetically a single disorder.

ACKNOWLEDGMENT

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REFERENCES


Mode of action of the IgG inhibitor of erythropoiesis in transient erythroblastopenia of children

EN Dessypris, SB Krantz, JS Roloff and JN Lukens