Congenital Hypoplastic Anemia Inhibition of Erythropoiesis by Sera From Patients With Congenital Hypoplastic Anemia

By Jorge A. Ortega, Nomie A. Shore, Peter P. Dukes, and Denman Hammond

An in vitro marrow culture assay designed to measure erythropoietic capability was used to ascertain the presence of an inhibitor in the sera of patients with congenital hypoplastic anemia (CHA). Marrow cells from nine anemic CHA patients responded to the stimulatory effect of exogenous erythropoietin (EPO) by an increase in heme synthesis in the presence of normal serum. The effect on heme synthesis was less than that observed with normal marrow cells. CHA serum inhibited heme synthesis by both normal and CHA marrow cells. It is concluded that an inhibitor of erythropoiesis is present in serum from CHA patients. This inhibitor most likely blocks the EPO-sensitive stem cell receptor sites, causing decreased response to the hormone.

CONGENITAL HYPOPLASTIC anemia (CHA) or Diamond-Blackfan syndrome is characterized by the presence of selective erythroid hypoplasia of unknown etiology, with high levels of serum erythropoietin (EPO). Smith in 1949 first suggested that this condition may be due to an immune defect but the presence of a circulating inhibitor to erythropoiesis has not been documented. Recently, Krantz has demonstrated the presence of a plasma inhibitor of heme synthesis in several adult patients with acquired pure red cell aplasia. Jepson and Lowenstein also reported the presence of a factor in the plasma gamma globulin fraction from a patient with aplastic anemia that inhibited the erythropoietin effect on erythropoiesis in polycythemic mice. Although immune therapy has been tried with some success in two children with CHA, an immune pathogenesis has not been established. The present study was designed to measure by an in vitro marrow culture assay the erythropoietic capability of patients with CHA and to ascertain the presence of an inhibitor of erythropoiesis.

MATERIAL AND METHODS

The in vitro bone marrow response to erythropoietin in the presence of normal AB positive serum or the patient’s own serum was studied in nine patients with CHA. All patients were anemic at the time of the study. Five nonanemic patients with cystic fibrosis (CF), one patient with idiopathic thrombocytopenic purpura (ITP), and one patient with acute glomerulonephritis served as controls. Hemoglobin, hematocrit, reticulocyte count, serum erythropoietin level, and bone marrow morphology were determined for each patient on the day of the marrow aspiration. The
nature of the study was explained to all patients and their parents and informed consent obtained for each patient studied.

The iron concentration of all control sera was adjusted to about 66% saturation to make it comparable to that of CHA sera.

For the short-term cell culture, 3-5 ml of bone marrow aspirate was transferred to 5 ml NCTC 135 medium containing 50 U/ml penicillin and 50 μg/ml streptomycin. One thousand units of heparin were added to the suspension. The cells were dispersed in the suspension by pipetting several times and then washed three times in the NCTC 135 medium. From the resultant sedimented cells, the buffy coat was pipetted off and suspended in 45 ml of a mixture containing human serum (20%) and NCTC 135 (80%). This cell suspension was divided into two 22.5-ml aliquots, and 1.2 U erythropoietin were added to one of the aliquots. The cells were transferred to 35-mm tissue culture dishes (Falcon Plastics) and incubated in a 5% CO₂-95% air atmosphere at 37°C. The total volume per dish was 0.8 ml, and the concentration of the cell suspension varied from 1.6 to $5.1 \times 10^6$ nucleated cells/ml.

After 42 hr incubation, $^{59}$Fe 0.45 μCi/dish was added. Four hours later, heme was extracted into cyclohexanone, and the incorporated $^{59}$Fe was measured according to the method previously described. All experiments were done in triplicate, and heme synthesis was expressed as the mean cpm of three dishes. With this method individual replicates do not differ by more than 10% from their means. Human urinary erythropoietin, specific activity 75.4 U/mg protein, was used as the exogenous erythropoietin. For the experiments with bone marrow from CHA patients, cells were separated in four different groups: (1) marrow cells incubated in normal AB positive serum, (2) marrow cells also incubated in normal AB positive serum, but each dish treated with 0.04 U of exogenous EPO, (3) marrow cells incubated in the presence of the patient's own serum, (4) marrow cells incubated in the presence of the patient's own serum and 0.04 U of exogenous EPO added. For the experiments with normal controls, cells were separated as above using serum from two patients with CHA (S.R. and K.K.) instead of the patient's own serum.

Erythropoietin activity in patient's serum was determined by the exhypoxic polycythemic mouse assay which measures the percent $^{59}$Fe uptake into circulating red cells. The assay method has been previously described in detail. In our laboratory normal nonanemic serum results in less than 1% $^{59}$Fe uptake. Serum EPO activity of the CHA patients used in this study ranged from 29% to 37% $^{59}$Fe uptake (approximately 2-4 IRP U/ml of serum).

Incorporation of the radioactive isotope by marrow cells in the presence of AB positive normal serum without EPO was defined as 100%. The method of statistical analysis was the t test.

**RESULTS**

As an initial step of the study, the effect of EPO on marrow cells from patients with nonhematologic disorders was studied. Cultured marrow cells from patients with CF in AB positive serum synthesized heme. These cells responded to exogenous EPO by an increase in heme synthesis. This increment was most apparent after 42 hr incubation (Table 1). Due to the variable cellularity between marrow specimens, the radioactive counts per minute differed widely. In order to make comparisons between the results from different marrow samples,
Table 2. Heme Synthesis After 42 Hours Incubation: Congenital Hypoplastic Anemia (Nine Patients)

<table>
<thead>
<tr>
<th>Patient</th>
<th>(1) Normal AB + Serum (cpm)</th>
<th>(2) EPO Added (cpm)</th>
<th>Difference (%)</th>
<th>(3) CHA Patients Serum (cpm)</th>
<th>(4) EPO Added (cpm)</th>
<th>Difference (%)</th>
<th>EPO Activity of Patients Serum (Per Cent $^{59}$Fe uptake)</th>
<th>Hg g/100 ml</th>
<th>Ref. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.R.</td>
<td>554</td>
<td>641</td>
<td>16</td>
<td>354</td>
<td>456</td>
<td>28</td>
<td>37.3</td>
<td>5.1</td>
<td>.2</td>
</tr>
<tr>
<td>J.J.</td>
<td>1910</td>
<td>2122</td>
<td>11</td>
<td>1985</td>
<td>1675</td>
<td>-16</td>
<td>36.4</td>
<td>7.7</td>
<td>1.1</td>
</tr>
<tr>
<td>A.C.</td>
<td>273</td>
<td>323</td>
<td>18</td>
<td>205</td>
<td>201</td>
<td>-2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D.T.</td>
<td>358</td>
<td>428</td>
<td>20</td>
<td>281</td>
<td>265</td>
<td>-6</td>
<td>—</td>
<td>6.9</td>
<td>0.2</td>
</tr>
<tr>
<td>J.F.</td>
<td>508</td>
<td>546</td>
<td>8</td>
<td>341</td>
<td>378</td>
<td>11</td>
<td>33.9</td>
<td>8</td>
<td>0.8</td>
</tr>
<tr>
<td>J.A.</td>
<td>176</td>
<td>214</td>
<td>22</td>
<td>201</td>
<td>142</td>
<td>-30</td>
<td>30.3</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>J.M.</td>
<td>869</td>
<td>847</td>
<td>-3</td>
<td>535</td>
<td>606</td>
<td>13</td>
<td>34.5</td>
<td>5.9</td>
<td>1.8</td>
</tr>
<tr>
<td>J.S.</td>
<td>354</td>
<td>440</td>
<td>24</td>
<td>382</td>
<td>373</td>
<td>-2</td>
<td>—</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>K.K.</td>
<td>857</td>
<td>866</td>
<td>1</td>
<td>800</td>
<td>746</td>
<td>-7</td>
<td>29.3</td>
<td>8.6</td>
<td>0</td>
</tr>
<tr>
<td>Normalized mean</td>
<td>100.0 ± 2.7%</td>
<td>111.5 ± 3.2%</td>
<td>11.5 ± 2.7</td>
<td>85.2 ± 4.4%</td>
<td>83.0 ± 3.1%</td>
<td>-2.2 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
data were normalized to per cent incorporation. When the results of EPO-treated cells were compared to non-EPO-treated controls and normalized, there was an increment of heme synthesis of 48% (SE ± 6.8%) in the EPO-treated group (Table 1).

Marrow cells from patients with CHA in the presence of normal AB positive serum responded to the stimulatory effect of exogenous EPO on heme synthesis (Table 2). The mean increment was 11.5% (SE ± 4.2%). This difference, although significant ($p < 0.01$), was less than that observed with CF marrow cells incubated under same conditions (Table 1). When the patients marrow cells were incubated in the presence of his own serum, the amount of heme synthesis was also not as great as when the same cells were incubated with normal AB positive serum (Fig. 1). The addition of exogenous EPO to marrow cells incubated in presence of the patient’s own serum resulted in no significant increase in heme synthesis over that obtained in the presence of normal AB positive serum or the patient’s serum without EPO.

Comparison of the results of cells cultured with added EPO (Fig. 1, bar graphs 2 and 4) showed the absence of an effect of exogenous erythropoietin on the marrow cells incubated in the presence of CHA serum ($p < 0.01$).

Marrow cell cultures from two nonanemic children were incubated with CHA serum and with control AB positive serum. As previously observed with marrow cells from CF patients, cells incubated in the presence of normal AB positive serum showed a significant stimulatory effect ($p < 0.01$) when exogenous erythropoietin was added (Table 3). The comparison of the two normal marrow cell cultures with added erythropoietin (Fig. 2, bar graphs 2 and 4) demonstrated the lack of EPO effect in the presence of CHA serum. The result

| Table 3. Heme Synthesis After 42 hr Incubation: Two Normal Controls |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Experiment | Normal AB Positive Serum (cpm) | EPO Added (cpm) | Difference (%) | CHA Patients Serum (cpm) | EPO Added (cpm) | Difference (%) |
| 1 | 7564 | 10260 | 36 | 7099 | 8141 | 14 |
| 2 | 3618 | 5424 | 50 | 3863 | 3423 | -11 |
| Normalized mean | 100.1 ± 5.1% | 137.8 ± 4.4% | 37.6 ± 5.6 | 97.2 ± 4.5% | 104.0 ± 6.0% | 6.8 ± 7.6 |

*For experiment 1, serum from patient S.E., and for experiment 2, serum from patient K.K. were used.
FIG. 2. In vitro heme synthesis by marrow cells from two normal controls. Differences between groups: 1 and 2, +37.8% ± 7.4%, p < 0.05; 3 and 4, +6.8% ± 7.6%, N.S.; 2 and 4, -33.8% ± 7.5%, p < 0.01; 1 and 3, -2.8% ± 6.8%, N.S.

DISCUSSION
It has been postulated that the pure red cell anemia of adults with thymoma is associated with an immune mechanism, since remissions have occurred frequently following removal of the tumor. Barnes suggested that there was a plasma inhibitor of erythropoiesis in such patients. Recently Krantz has demonstrated the presence of an antibody or immune complex in the sera of patients with acquired pure red cell anemia which was cytotoxic for erythroblasts.

The response to exogenous EPO in the in vitro short-term suspension culture of marrow cells from nonanemic children with CF demonstrated that heme synthesis as measured by the incorporation of 59Fe into heme continued after removal from the host. In the presence of normal type AB Rh-positive serum, 59Fe uptake was appreciable after 42 hr of the marrow culture. This suggested that 59Fe uptake was the result of heme production by erythroblasts maturing at the time of marrow sampling as well as by erythroblasts formed from EPO-responsive stem cells present in the marrow sample.

Marrow cells from patients with CHA responded to exogenous EPO in the presence of normal AB positive serum. However, the response was significantly less than that obtained with marrow from CF patients. This defective response of CHA marrow to exogenous EPO may be similar to that described in the W/WV mouse. In the latter animal, 150 times more EPO was required to elicit an effect than in the normal mice. Despite the limited response to exogenous EPO, W/WV erythropoiesis appeared to be regulated by EPO, since plasma EPO of such mice were slightly higher than normal. Furthermore, W/WV mice treated with antierythropoietin serum showed a decrease in marrow erythroid cells with no change in numbers of marrow spleen colony-forming units.

Likewise, erythropoietin in CHA patients would appear to be operative in erythropoiesis, since EPO stimulated heme synthesis in vitro.

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there is a linear relationship between the degree of anemia and the serum EPO level of CHA patients, suggesting a direct relationship between the hypoxic stimulus, marrow erythroid activity, and erythropoietin.

The nature of the defect in the W series mouse does not appear to be due either to a lack of erythroid-committed stem cells or to a plasma inhibitor of EPO. Similarly patients with CHA in spontaneous or corticosteroid-induced remission have normal erythroid marrow, suggesting adequate erythroid stem cells. However, impaired response to EPO by erythroid stem cells of the W series mouse or the CHA patient might be accounted for if it were postulated that their erythropoietin-responsive stem cells contained fewer receptor sites for EPO, or if some of these receptor sites were inoperative.

Minute but significant numbers of inhibitor molecules may have remained attached to receptor sites on the CHA marrow cells after the washing and consequent incubation in normal serum. These blocked receptor sites could account for the impaired response of CHA cells as compared to CF cells, even when incubated with normal serum.

The suppression of synthesis by normal marrow cells in CHA serum would tend to confirm the presence of an inhibitor. The effect of exogenous EPO was abolished in this system. Since the CHA serum contained at least tenfold more EPO than was added, the inhibitor does not seem to act by neutralizing or combining with EPO, but by blocking EPO receptor sites of erythropoietin-responsive cells. Indirect evidence for this explanation might be drawn from the observation that in the intact assay animal CHA anemic serum produces a marked erythropoietic effect; the inhibitor was either nullified in some way or was present in insufficient amounts to block all the receptor sites on marrow cells. An alternative explanation for the lack of inhibitory effect of CHA serum in the test mouse or the rat marrow in vitro assay would be to postulate that the inhibitor is specific for human marrow. In the CHA patient whose erythropoietin-responsive cells may contain a deficient number of receptor sites, the inhibitor would suppress the action of erythropoietin. This inhibitor may very well result in damage to the EPO-sensitive stem cell by blocking receptor sites, causing the limited response to exogenous EPO observed in vitro and the lack of active bone marrow erythropoiesis in patients with CHA.

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