Diphenylhydantoin-Induced Pure Red Cell Aplasia

By Emmanuel N. Dessypris, Susan Redline, John W. Harris, and Sanford B. Krantz

The pathogenesis of diphenylhydantoin-induced pure red cell aplasia was investigated in the case of a 32-year-old man who developed pure red cell aplasia while he was under treatment with diphenylhydantoin. The patient’s serum IgG purified from serum drawn at the time of diagnosis suppressed normal allogeneic marrow colony-forming (CFU-E) and burst-forming (BFU-E) and autologous blood BFU-E growth in vitro only in the presence of diphenylhydantoin. This IgG–diphenylhydantoin complex had no effect on CFU-GM growth in vitro. Normal IgG or patient’s IgG purified from serum drawn after the remission of red cell aplasia had no effect on erythroid colony formation in vitro in the presence of diphenylhydantoin. The IgG–diphenylhydantoin complex exerted no direct cytotoxic effect on normal marrow erythroblasts, CFU-E, and BFU-E, nor did it interfere with the action of erythropoietin on marrow erythroblasts. These studies suggest that diphenylhydantoin-induced red cell aplasia is immunologically mediated through an IgG inhibitor, which requires the presence of the drug to suppress erythroid colony formation in vitro. This inhibitor seems to exert its effect on erythroid progenitors at or beyond the stage of differentiation of CFU-E, but not on erythroblasts.

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PCV was 19% with normochromic normocytic indices, and his reticulocyte count was 0.1%. His WBC, differential, and platelet counts were normal. Serum haptoglobin, folate, and B12 were normal. An antitrypsin test was negative. Serum ferritin was 1,217 ng/mL, SGOT 135 U/mL, SGPT 217 U/mL, and bilirubin 0.2 mg/dL. A bone marrow aspirate and biopsy revealed almost complete absence of erythroid precursors, with normal myelopoiesis, normal megakaryocytes, and a few lymphoid aggregates. The rare erythroid precursors present showed heavy basophilic granulation and karyorrhexis. A diagnosis of PRCA was made and the diphenylhydantoin was discontinued. A computerized tomogram of the chest was negative for thymoma. The patient was transfused with RBCs to a PCV of 30% and discharged. One week later, his PCV rose to 35% and his reticulocyte count rose to 6.7%. Within the following two weeks, his PCV normalized to 44% and has since remained within the normal range.

In Vitro Studies

Blood was drawn from the patient at the time of diagnosis of PRCA and before any RBC transfusions were given, as well as six weeks later when the PRCA had remitted. Serum was collected and stored at −80°C before it was used. Serum IgG was purified from the patient’s sera and from the pooled sera of 20 type AB normal donors by ammonium sulfate precipitation followed by diethylaminoethanol (DEAE)-cellulose ion exchange chromatography. The final preparation was extensively dialyzed in α-modification of Eagle’s minimum essential medium (α-MEM) and sterilized by filtration. These IgG preparations were free of impurities demonstrable by immunoelectrophoresis, using antisera against whole human serum, against human immunoglobulins, and γ-chain-specific antisera (Meloy Laboratories, Springfield, Va). IgG purified from serum drawn at diagnosis is designated as IgG-I and that purified from serum drawn after remission as IgG-II. Diphenylhydantoin sodium solution in water (10 mg/mL) was prepared immediately before it was used and was sterilized by filtration.

Bone marrow cells from normal adult volunteers and blood from the patient were collected in preservative-free heparin and diluted with an equal volume of Iscove’s modification of Dulbecco’s MEM (IMDM). Light-density nonadherent cells were separated by centrifugation over Ficoll-Hypaque (density, 1.077) and adherence to plastic. Bone marrow cells were cultured for erythroid colonies at a concentration of 10⁶/0.2 mL of medium, and blood cells at a concentration of 4 × 10⁵/0.5 mL of medium. Myeloid colonies were cultured at a concentration of 4 × 10⁵/mL of medium—CFU-E and BFU-E were assayed by a modification of the plasma clot method of McLeod et al. The medium consisted of 1% MEM with 30% heat-inactivated human AB serum, 0.2% deionized human serum albumin, 120 μg of human fibrinogen per milliliter, 100 nM β-mercaptoethanol, 200 units of penicillin per milliliter, 20 mg of streptomycin per milliliter, and human urinary erythropoietin (1,140 U/mg; CAT-1, National Institutes of Health, Bethesda, Md), or sheep plasma erythropoietin (2.16 U/mg; Connaught Laboratories, Willowdale, Ontario, Canada) at a final concentration of 1 and 3 U/mL for the growth of CFU-E and BFU-E, respectively. Clotting was induced by adding 0.2 units of bovine thrombin per milliliter and 0.1 mL of bovine citrated plasma. The effect of IgG on erythroid colony formation in vitro was tested by replacing a volume of α-MEM with an equal volume of IgG preparation. Before the clot formed, 0.2 mL of medium was transferred to 0.25 mL wells in a disposable microtiter tray. For blood BFU-E, 0.5 mL of medium was transferred to 1.7 × 1.6 cm-wells in a multiwell tissue culture plate. The cells were incubated at 37°C in a highly humidified atmosphere of 5% CO₂ for seven to 15 days. The clots were then removed, fixed, and stained with benzidine and hematocytin, as described by McLeod et al. CFU-E were enumerated on day 7 by counting all benzidine-positive colonies containing eight or more cells. BFU-E were enumerated on day 15 according to the criteria of Clarke and Housman. For quantitation of CFU-E and BFU-E, four to six clots were counted, and the results are expressed as mean ± SEM. Previous experiments in this laboratory have established a linear relationship between CFU-E/BFU-E growth and the number of plated cells at concentrations of 0.5 × 10⁴ to 4 × 10⁶ cells per milliliter. The growth of CFU-E and BFU-E in vitro by this method reaches a plateau at concentrations of 1 and 3 units of erythropoietin per milliliter, respectively.

CFU-GM were assayed by the methylcellulose method of Iscove et al. The medium consisted of α-MEM with 0.8% methylcellulose, 20% fetal calf serum, and 1% deionized bovine serum albumin. The effect of IgG on myeloid colony formation in vitro was tested by replacing a volume of α-MEM by an equal volume of IgG preparation. Colony-stimulating activity was provided by the addition of medium conditioned by peripheral blood lymphocytes stimulated with phytohemagglutinin in the presence of 1% bovine albumin for five days. This conditioned medium has a specific activity of 978 U/mL and provides maximum growth when added at a concentration of 10% (vol/vol). A linear relationship between the numbers of CFU-GM and plated cells has been established previously in this laboratory. Aliquots (0.5 mL each) of the medium and cells were transferred to wells, 1.7 × 1.6 cm, in a multiwell tissue culture tray and incubated for ten days at 37°C in a highly humidified atmosphere of 5% CO₂. At the end of the incubation period, clotting was induced by the method of McMahon and Hankins, and the clots were fixed with glutaraldehyde and stained with Wright’s stain. Colonies of 50 or more cells containing granulocytes, eosinophils, or macrophages, or any mixture of them, were enumerated. In normal marrows, >90% of the colonies consist of granulocytes and/or macrophages. All experiments were run in quadruplicate, and the results are expressed as mean ± SEM.

Pretreatment of bone marrow or blood mononuclear cells with IgG, diphenylhydantoin, and complement was performed as previously described. In summary, 4 × 10⁴ cells in 0.1 mL α-MEM or 0.1 mL solution of IgG in α-MEM (final concentration, 4 mg/mL) with or without diphenylhydantoin sodium (1.5 μg/mL), in the presence of 0.3 mL fresh AB human serum as a source of complement, were incubated for one hour at 37°C. At the end of incubation, the cells were washed three times with 2 mL of α-MEM and resuspended in 0.2 mL IMDM. One-tenth milliliter of this cell suspension was transferred to culture medium and assayed for CFU-E and BFU-E.

Heme synthesis in vitro by bone marrow erythroblasts was assayed as previously described. In short, 5 × 10⁴ unseparated bone marrow cells were transferred to 1 mL of medium containing 30% NCTC-109, 30% α-MEM, 20% heat-inactivated normal AB or patient’s serum, 20% fetal calf serum, and 0.25 units of erythropoietin and incubated at 37°C in a 5% CO₂ atmosphere with high humidity. After 40 hours of incubation, 0.3 μCi of ⁵⁹Fe as ferric chloride, previously bound to human transferrin, was added to each tissue culture dish. The incubation was continued for 24 hours, when the contents of the dishes were transferred to tubes; heme was extracted by cyclohexanone and counted in an automatic gamma scintillation counter.

Erythroblast cytotoxicity assay was performed according to the method of Krantz and Zaentz. In summary, bone marrow erythroblasts labeled with ⁵⁹Fe were initially incubated with normal or patients’ IgG (4 mg/mL) with or without diphenylhydantoin (1.5 μg/mL) for one hour at 37°C, and then washed twice with 2 mL of α-MEM before being transferred to tubes containing 1 mL of α-MEM with 30% fresh human AB serum as a source of complement. After 20 hours of incubation at 37°C in a 5% CO₂ highly humidified atmosphere, the tubes were centrifuged, the supernatants...
were collected, and both pellets and supernatants were counted in an automatic gamma scintillation counter. The results were expressed as a release index (RI).

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RI = \frac{\text{Radioactivity of the supernatant} \times 100}{\text{Total radioactivity}}
\]

It has previously been demonstrated that the \(^{59}\text{Fe}\) radioactivity in the supernatant represents loss of radioactive hemoglobin from the erythroblasts and is an index of erythroblast cytotoxicity.\(^9\)

The presence or absence of diphenylhydantoin in the IgG-I preparation was tested by the fluorescence polarization immunoassay\(^8\) and by gas chromatography (kindly performed by Dr K.H. Dudley). In addition to determining whether IgG-I and -II were capable of forming a complex with diphenylhydantoin, 15 \(\mu\)g of this drug was added to 1 mL of IgG preparations (40 mg/mL) and diphenylhydantoin was subsequently assayed by gas chromatography and by the fluorescence polarization immunoassay.

All studies were approved by the Vanderbilt University and the Cleveland Metropolitan General Hospital Committees for the Protection of Human Subjects.

RESULTS

When normal human marrow cells from three donors were cultured in the presence of the patient’s IgG (4 mg/mL), purified from serum drawn at the time of diagnosis of PRCA (IgG-I), a significant suppression of CFU-E and BFU-E growth in vitro was noted only in the presence of diphenylhydantoin \((P < .02 \text{ for CFU-E} \text{ and } P < .01 \text{ for BFU-E})\) (Fig 1). The same concentration of the patient’s IgG-I and diphenylhydantoin had no effect either on the number or the type of normal CFU-GM–derived colonies in vitro. Diphenylhydantoin per se had no effect on erythroid and myeloid colony formation in vitro at the concentrations used in these experiments (1.5 \(\mu\)g/mL). Under the same culture conditions, no suppression of erythroid colony formation in vitro could be detected in the presence of either normal or patient’s IgG purified from serum drawn after remission of PRCA (IgG-II).

In cultures containing IgG-I and diphenylhydantoin, but not in those containing IgG-I alone, normal IgG, or IgG-II with or without diphenylhydantoin, an increased number of erythroid colonies consisting of one to five erythroblasts was noted. There were 34 ± 8 (average ± SEM from two experiments) such colonies in cultures containing IgG-I and diphenylhydantoin and only 13 ± 5 such colonies in control cultures. Whether these day 7 erythroid colonies represented “defective or abortive” CFU-E–derived colonies is not known.

Patient’s IgG-I (2 mg/mL) also exerted a similar effect in the presence of diphenylhydantoin on autologous blood BFU-E (Fig 2). Furthermore, the addition of IgG and diphenylhydantoin to the culture medium on the eighth day of culture resulted in an equal degree of suppression of erythroid colony formation, indicating that the IgG and diphenylhydantoin combination exerted its effect on erythroid progenitors at a stage of differentiation equal to, or more mature than, the CFU-E.

Preincubation of normal human marrow cells for one hour at 37 \(^\circ\)C, with normal or patient’s IgG-I, diphenylhydantoin, and fresh normal serum as a
source of complement, followed by repeated washing and assay of the marrow cells for CFU-E and BFU-E, resulted in no significant decline in the amount of CFU-E and BFU-E growth in vitro (P > 0.05) (Table 1). These experiments indicated that the patient's IgG and diphenylhydantoin exerted no direct cytotoxic effect upon normal CFU-E and BFU-E in vitro. Similar experiments performed on autologous blood BFU-E failed to demonstrate any direct cytotoxic effect of the patient's IgG, diphenylhydantoin, and complement on patient's blood BFU-E (data not shown).

When normal marrow erythroblasts were labeled with $^{59}$Fe and were then exposed to the patient's IgG, diphenylhydantoin, and fresh human AB serum as a source of complement, the release of $^{59}$Fe in the supernatant following 20 hours of incubation at 37 °C was equal to the release of $^{59}$Fe measured in the presence of normal IgG, indicating the absence of a direct cytotoxic effect of the IgG-diphenylhydantoin combination on normal mature erythroid cells (Table 2). In addition, normal bone marrow cells in short-term liquid culture responded to erythropoietin equally well in the presence of diphenylhydantoin and normal or patient's serum (Table 3), indicating that the erythropoietin inhibitor in the patient's serum did not interfere with the action of erythropoietin on normal marrow erythroid precursors.

Diphenylhydantoin was undetectable in the IgG-I preparation either by the fluorescence polarization immunoassay or by gas chromatography. In addition, both methods detected the same amount of added diphenylhydantoin in the patient's IgG-I preparation, indicating the lack of any significant affinity of IgG-I for diphenylhydantoin.

**DISCUSSION**

Drug-induced PRCA represents an acute and generally reversible form of isolated erythroid aplasia. Recovery of erythropoiesis usually occurs shortly after discontinuation of the offending medication. The patient presented in this report was exposed to two different drugs that have been previously reported to cause PRCA: isoniazid and diphenylhydantoin. Isoniazid, however, was discontinued three weeks before the diagnosis of PRCA, and the possibility that isoniazid was responsible for PRCA is very unlikely, since erythroblastopenia and reticulocytopenia persisted and the patient's clinical condition deteriorated during the three weeks following its withdrawal. The patient's prompt hematologic recovery following discontinuation of diphenylhydantoin and the results of the in vitro studies suggest that in this case diphenylhydantoin was the agent responsible for induction of the erythroid aplasia. The association of diphenylhydantoin with PRCA has been well documented by the work of Brittingham et al., who rechallenged their patient with diphenylhydantoin many times and were able to induce erythroid aplasia or hypoplasia that was always easily reversible upon cessation of the drug. The results of our studies in vitro demonstrate that erythroid aplasia in this case may have been induced by the serum IgG in the presence of diphenylhydantoin. This combination of IgG plus diphenylhydantoin suppressed normal allogeneic marrow CFU-E and BFU-E and autologous blood BFU-E growth in vitro without affecting the growth of CFU-GM. However, no direct toxicity of the IgG plus diphenylhydantoin could be detected against normal marrow BFU-E, CFU-E, or erythroblasts, or against autologous control BFU-E. In addition, the IgG–diphenylhydantoin combination did not interfere with the action of erythropoietin on normal marrow erythroid cells. Our studies

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**Table 1. Effect of Pretreatment of Marrow Cells With Diphenylhydantoin and Normal or Patient's IgG and Complement**

<table>
<thead>
<tr>
<th>Marrow Cells Pretreated With</th>
<th>CFU-E/10^6 Cells</th>
<th>BFU-E/10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MEM</td>
<td>165 ± 19</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>143 ± 23</td>
<td>51 ± 3</td>
</tr>
<tr>
<td>Normal IgG and DPH</td>
<td>171 ± 26</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>Normal IgG, DPH, and C'</td>
<td>159 ± 13</td>
<td>53 ± 5</td>
</tr>
<tr>
<td>Patient's IgG</td>
<td>148 ± 21</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>Patient's IgG and DPH</td>
<td>161 ± 24</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>Patient's IgG, DPH, and C'</td>
<td>167 ± 18</td>
<td>49 ± 8</td>
</tr>
</tbody>
</table>

IgG was added at a final concentration of 4 mg/mL and diphenylhydantoin (DPH) at 1.5 μg/mL. Numbers represent the means ± SEM from two experiments run in quadruplicate. C', complement provided by fresh AB human serum.

**Table 2. Erythroblast Cytotoxicity Assay**

<table>
<thead>
<tr>
<th></th>
<th>$^{59}$Fe Release Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal IgG</td>
<td>13.07 ± 0.81</td>
</tr>
<tr>
<td>Normal IgG and DPH</td>
<td>14.10 ± 0.94</td>
</tr>
<tr>
<td>Normal IgG, DPH, and C'</td>
<td>13.40 ± 0.68</td>
</tr>
<tr>
<td>Patient's IgG</td>
<td>13.47 ± 1.21</td>
</tr>
<tr>
<td>Patient's IgG and DPH</td>
<td>13.89 ± 0.69</td>
</tr>
<tr>
<td>Patient's IgG, DPH, and C'</td>
<td>13.80 ± 1.13</td>
</tr>
</tbody>
</table>

IgG was added at a final concentration of 4 mg/mL and diphenylhydantoin at 1.5 μg/mL. Numbers represent means ± SEM from two experiments run in quadruplicate.

**Table 3. Response of Normal Marrow Cells to Erythropoietin in the Presence of Normal or Patient's Serum With or Without Diphenylhydantoin**

<table>
<thead>
<tr>
<th></th>
<th>$^{59}$Fe-Heme (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>743 ± 102</td>
</tr>
<tr>
<td>Normal serum and EP</td>
<td>2,319 ± 315</td>
</tr>
<tr>
<td>Normal serum, EP, and DPH</td>
<td>2,513 ± 268</td>
</tr>
<tr>
<td>Patient's serum</td>
<td>514 ± 82</td>
</tr>
<tr>
<td>Patient's serum and EP</td>
<td>1,905 ± 213</td>
</tr>
<tr>
<td>Patient's serum, EP, and DPH</td>
<td>2,118 ± 294</td>
</tr>
</tbody>
</table>

Numbers represent means ± SEM from two experiments run in quadruplicate. EP, erythropoietin (0.25 U/mL); DPH, 1.5 μg/mL.
suggest that the target cell(s) for this inhibitor is an erythroid progenitor at or beyond the stage of differentiation of the CFU-E, but not the differentiated erythroblasts. Although the significance of erythroid colonies consisting of fewer than eight erythroblasts on day 7 in culture is not known, the presence of an increased number of such colonies—possibly representing "defective or abortive" CFU-E—only in cultures containing IgG-I and diphenylhydantoin is also consistent with the view that the target cell for this inhibitor is in a phase of differentiation between CFU-E and proerythroblast. However, whether this inhibitor acted through cytotoxicity or a block of differentiation is not known. It is also conceivable that this inhibitor acted not directly on erythroid cells but indirectly through an effect on an accessory cell necessary for the differentiation of this erythroid progenitor to mature erythroblasts. This hypothesis, however, seems unlikely, since at this stage of erythroid differentiation there is no evidence for a primary role of such an accessory cell.

The interaction between patient’s IgG and diphenylhydantoin is not known. Neither IgG alone nor diphenylhydantoin alone was capable of suppressing erythropoiesis in vitro. It is conceivable that diphenylhydantoin formed an immune complex with the patient’s IgG-I. However, we were unable to detect any diphenylhydantoin in the patient’s purified serum IgG-I fraction by the use of either the fluorescence polarization immunoassay or gas chromatography. Furthermore, the addition of diphenylhydantoin to the patient’s IgG-I solution did not interfere with the fluorescence polarization immunoassay, indicating that if the IgG has any affinity for diphenylhydantoin this must be very weak, or directed to an antigenic site of the molecule different from the one to which the commercially available antibody used in the immunoassay is directed.

The pathogenesis of diphenylhydantoin-induced PRCA has been the subject of two previous investigations. Yunis et al. studied the case reported by Brittingham et al. and demonstrated that diphenylhydantoin in that patient exerted its toxic effect by specifically inhibiting DNA synthesis in erythroid cells probably at the step of deoxyribonucleotide formation. These studies were performed in the presence of patient’s serum and do not exclude the possibility that this effect was mediated through an interaction of patient’s IgG with diphenylhydantoin. Based on their clinical observations, these investigators suspected an immunologic mechanism; however, they were unable to demonstrate any increased amount of human immunoglobulin on the patient’s marrow erythroblasts by the use of fluorescein-labeled rabbit antihuman globulin. This negative result is in agreement with the results of our studies, demonstrating no effect of the IgG–diphenylhydantoin combination on differentiated marrow erythroid precursors. A possible immune mechanism was finally considered to be unlikely in the same case, because infusion of patient’s serum, 500 mL/d for three consecutive days, to a normal volunteer treated with diphenylhydantoin did not result in any change of the recipient’s erythropoiesis as judged by his reticulocyte count, number of erythroblasts in the marrow, serum iron, iron-binding capacity, or 59Fe clearance. However, since the target cell for the IgG–diphenylhydantoin complex is not the marrow erythroblast but an earlier cell between the CFU-E and proerythroblast, one would not expect any prompt change of reticulocyte count, number of marrow erythroblasts, or 59Fe clearance, unless an adequate concentration of the responsible IgG was maintained in the recipient’s blood for a period of at least five to seven days, which would be needed to exhaust the marrow reserve of already differentiated erythroid precursors and result in diminished erythropoiesis and reticulocytopenia.

Another case of diphenylhydantoin-induced PRCA and autoimmune hemolytic anemia has been studied by Lee et al. These investigators, using the Marbrook chamber technique for growing erythroid cells in vitro, were able to demonstrate the presence of a toxic factor in their patient’s serum, which appreciably reduced the number of erythroblast foci. Such a factor was not detectable in serum drawn after remission of PRCA.

The demonstration of an immune mechanism responsible for the diphenylhydantoin-induced red cell aplasia is not surprising, since many cases of PRCA appear to be immunologically mediated and since diphenylhydantoin has been associated with various abnormalities of the immune system. Whether diphenylhydantoin in this case acted primarily as a hapten, thus inducing a normal immune response, or in addition altered the patient’s immune function to the point of allowing production of an autoantibody is not known. However, the fact that diphenylhydantoin is a membrane-active agent and its presence was necessary to induce suppression of erythropoiesis in vitro is in favor of its primary role as a hapten.

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