Erythroid Failure in Diamond-Blackfan Anemia Is Characterized by Apoptosis

By Eva B. Perdahl, Beth L. Naprstek, William C. Wallace, and Jeffrey M. Lipton

Programmed cell death, also known as apoptosis, is frequently initiated when cells are deprived of specific trophic factors. To investigate if accelerated apoptosis contributes to the pathogenesis of Diamond-Blackfan anemia (DBA), a rare pure red blood cell aplasia of childhood, we studied the effect of erythropoietin (epo) deprivation on erythroid progenitors and precursors from the bone marrow of DBA patients as compared with hematologically normal controls. Apoptosis in response to epo deprivation was evaluated by enumeration of colony-forming unit-erythroid (CFU-E) and burst-forming unit-erythroid (BFU-E) derived colonies in plasma clot semisolid culture and by the identification of typical DNA oligosomes by gel electrophoresis from marrow mononuclear cells in liquid culture. In all DBA patients there was a marked decrease in CFU-E- and BFU-E-derived colony formation compared with normal controls at comparable time points of epo deprivation, with a complete loss of CFU-E-derived colonies in semisolid culture by 9 hours of epo deprivation versus 48 hours in controls. The BFU-E-derived colony response to epo deprivation displayed a similar pattern of decrement. Apoptotic changes assessed by the presence of characteristic DNA fragmentation began in the absence of epo deprivation and were readily detected within 3 hours of epo deprivation in DBA cultures versus 9 hours in controls. We conclude that DBA is characterized by accelerated apoptosis as measured by the loss of erythroid progenitor clonogenicity and increased progenitor and precursor DNA fragmentation leading to the formation of characteristic oligosomes, consistent with an intrinsic erythroid-progenitor defect in which increased sensitivity to epo deprivation results in erythroid failure.

DIAMOND-BLACKFAN anemia (DBA) is a rare pure red blood cell (RBC) aplasia of childhood characterized by moderate to severe anemia, reticulocytopenia, macrocytosis, and in the vast majority of cases, a paucity of bone marrow (BM) erythroid precursors. There is usually no leukopenia or thrombocytopenia with normal precursors of these elements present in the BM. The syndrome is associated with a variety of somatic malformations in less than 30% of cases. Since the first description of DBA over 50 years ago, there have been a number of theories put forth regarding its etiology. Recent investigators have suggested humoral or cellular suppression of erythropoiesis, a microenvironmental defect, a block in maturation between the multipotent myeloid progenitor and the earliest erythroid progenitor, accessory cell failure, and an intrinsic progenitor defect. Despite reports of immunologic abnormalities and microenvironmental or accessory cell defects in some patients with DBA, the consensus among investigators is that the majority of cases appear to result from an intrinsic disorder of the erythroid progenitor that involves its inability to respond normally to inducers of erythroid proliferation and differentiation. The most thoroughly studied inducers include erythropoietin (epo), interleukin-3 (IL-3), and stem cell factor. Of particular note, no receptor-ligand defects have yet been identified. Thus, the specific nature of what is probably a heterogeneous disorder, remains unknown.

Recent work by Khoury and Bondurant note that the previously reported loss of clonogenicity of normal erythroid progenitors in semisolid culture, in proportion to the duration of epo deprivation, is a likely consequence of apoptosis. Based on these observations, the current study was undertaken to test the hypothesis that DBA is characterized by erythroid progenitors and precursors highly susceptible to apoptosis, as demonstrated by the accelerated loss of erythroid progenitor clonogenicity, and the concomitant appearance of typical DNA oligosomes in the face of epo deprivation.

MATERIALS AND METHODS

Subjects. BM was obtained after informed consent from the posterior iliac crest of seven patients (Table 1) who fulfilled the diagnostic criteria for DBA. Patients LV, ML, JK, and PM ages 16, 20, 5 1/2, and 34 years respectively, were chronically transfused and off steroids, and patients YD, and JB ages 4 1/2 and 4 10/12 years respectively, had been receiving steroid treatments at the time of the study. Patient OT, age 15 years, was receiving IL-3 at the time of study.

Patient LV, female, was diagnosed at 7 weeks of age. Initially treated with prednisone, the therapy was discontinued because of

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severe growth retardation, osteopenia and ultimately steroid refractoriness. She had been on chronic red blood cell transfusions and desferal chelation, in good iron balance, for 6 years at the time of the study. She has multiple skeletal abnormalities including a shortened metacarpal bone and distal phalanx of the left thumb. There was consanguinity five generations ago and the patient and her brother both have ocular albinism unrelated to DBA. ML, male, was diagnosed at six weeks of age. He was initially treated daily, then on alternate days, with prednisone resulting in a good response. A progressive decrease in steroid responsiveness followed requiring increasing doses until age 13 years when steroid treatment was discontinued because of aseptic necrosis of the calcaneus. He had been on chronic RBC transfusions and desferal chelation, in good iron balance, for 7 years at the time of the study. He has short stature but is otherwise normal. Patient JK, male, was diagnosed at 5 weeks of age. The patient never responded to prednisone and therefore, is chronically transfused and on desferal chelation with moderate iron balance. He has a hypoplastic left hand with five underdeveloped digits, a decreased radial pulse, and a symmetrically short left forearm, consistent with amniotic band syndrome, felt to be unrelated to DBA. Patient PM, female was diagnosed at 2 1/2 years of age. She responded initially to prednisone and was tapered to an every-other-day dose. She became anemic when steroids were discontinued and she received her first transfusion at 24 years of age. Prednisone was reinstituted and she did well until she was 31 years old when she became gradually RBC-transfusion dependent. Her physical exam, other than for mild corticosteroid effects, was unremarkable. YD, male, was diagnosed at 6 weeks of age. He was initially treated daily with methylprednisolone showing no improvement. He required 6 U of packed RBCs during his first year of life but has not been transfused since. Over the last three years, he has responded to oral prednisolone pulses of 30 mg/d for 3 days every 10

**Table 1. Clinical Characteristics of Patients With DBA**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Presentation</th>
<th>Sex</th>
<th>Age at Study</th>
<th>CBC at Presentation</th>
<th>BM at Presentation</th>
<th>Steroid Response</th>
<th>PRBC Transfusions</th>
<th>Treatment at Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV</td>
<td>4 wks F</td>
<td>17</td>
<td>Hb 2.3</td>
<td>Erythroid hypoplasia</td>
<td>Yes*</td>
<td>Multiple Transfusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML</td>
<td>4 wks M</td>
<td>21</td>
<td>Hb 3.2</td>
<td>Erythroid hypoplasia</td>
<td>Yes*</td>
<td>Multiple Transfusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JK</td>
<td>5 wks M</td>
<td>51</td>
<td>Hb 3.7</td>
<td>Erythroid hypoplasia</td>
<td>No</td>
<td>Multiple Transfusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>2 1/2 yrs F</td>
<td>34</td>
<td>Hb 6.6</td>
<td>Erythroid hypoplasia</td>
<td>Yes*</td>
<td>Multiple Transfusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>2 1/2 yrs F</td>
<td>34</td>
<td>Hb 6.6</td>
<td>Erythroid hypoplasia</td>
<td>Yes</td>
<td>6 Prednisolone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YD</td>
<td>6 wks M</td>
<td>41/2</td>
<td>Hb 8.0</td>
<td>Erythroid hypoplasia</td>
<td>Yes</td>
<td>None Prednisone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>2 1/2 yrs M</td>
<td>35</td>
<td>Hb 4.0</td>
<td>Erythroid hypoplasia</td>
<td>Yes</td>
<td>Multiple IL-3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Initial steroid response followed by a progressive decrease in response resulting in dose increments leading to steroid refractoriness (LV, PM) and/or severe toxicity (ML, LV).

**Abbreviations:** CBC, complete blood count; PRBC, packed red blood cell; NA, not available.

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**Fig 1.** (A) Influence of delayed epo on BM CFU-E colony formation in DBA versus controls. Data are from two patients with DBA (LV, YD) compared with the mean ± SEM of four controls. Percentage of maximum CFU-E colony frequency is expressed as a function of the time of addition of epo to the cultures. Actual colony counts are enumerated in Table 2. The t_{1/2} max (time of epo deprivation leading to half-maximal colony expression) for CFU-E colony formation was approximately 3 hours for the DBA patients versus 9 hours for the controls. (B) Influence of delayed epo addition on BM BFU-E colony formation in DBA versus controls are presented as in (A) for BFU-E-derived colony formation. Actual colony counts are enumerated in Table 3. The t_{1/2} max for BFU-E colony formation was approximately 3 hours for DBA versus 15 hours for controls.
to 12 days. He is developmentally delayed, walking at 19 months and not yet speaking in sentences at age 4 years. Patient JB, male, was diagnosed at 18 months of age. He responded to prednisone and is currently responding to an every-other-day schedule. His physical exam is unremarkable. Patient OT, female, was diagnosed at 12 weeks of age. She failed to respond to prednisolone and was placed on a RBC-transfusion regimen with desferal chelation. She had a splenectomy at 11 years of age. At age 11 years, a trial of methyl-prednisolone was successful and she remained on low-dose daily therapy until responding to IL-3 at approximately 16 years of age. She is off prednisone and IL-3 and on a RBC-transfusion regimen with desferal chelation. She had a hematologic remission.

Normal control BM was obtained after informed consent from the posterior iliac crest of four healthy donors ages 25 to 27 years. Normal control BM was obtained after informed consent from the posterior iliac crest of four healthy donors ages 25 to 27 years. Plasma clot culture. DBA and normal BM low-density mononuclear cells (MNC) prepared by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation were plated at 2 × 10^5 cells/mL clot suspension in the plasma clot system previously described and modified. Briefly, clotting was initiated by the addition of 0.1 U of grade I bovine thrombin (Sigma Chemicals, St Louis, MO). The 1.0-mL clotting mixture was dispensed in 0.1-mL aliquots into 0.2-mL microtitre culture wells (Linbro plates, Linbro Scientific, New Haven, CT) and incubated under 5% CO2 in high humidity. The culture system contained 2 U/mL recombinant human epo (Amgen) and 0.2 U in 0.008 mol/L EDTA as running buffer. After 2 to 7 days in culture, cell viability was confirmed by trypan blue exclusion.

DNA extraction. DNA extraction was performed by either phenol/chloroform or high-salt extraction. In phenol/chloroform extraction, harvested cells were resuspended in extraction buffer (150 mmol/L NaCl, 10 mmol/L EDTA pH 8.0, 10 mmol/L Tris pH 7.5 and 0.1% sodium dodecyl sulfate [SDS]). RNAse A (1 mg/mL), (Sigma) was added and the sample incubated for 0.5 to 3 hours at 37°C. Proteinase K (2 mg/mL) (Sigma) was added to the sample for digestion at 37°C overnight. The phenol/chloroform extraction was performed as described by Maniatis et al. For high-salt extraction, DNA was extracted as described by Miller et al with the addition of RNAse A digestion before the addition of Proteinase K. No difference in DNA quantity or quality was noted between the two extraction methods (data not shown). The addition of RNAse A incubation to remove RNA contamination from this assay does not compromise the DNA quality (data not shown). The amount and purity of DNA obtained was quantified by A260,280 in a Beckman DU-64 Spectrophotometer (Beckman Instruments, Pasadena, CA).

Polyacrylamide gel electrophoresis (PAGE). Acrylamide solution for 0.75 mm thick 7 mol/L Urea, 8% polyacrylamide gel (acrylamide: bis-acrylamide: bis-acrylamide 19:1) was prepared as described by Davis et al with 1 × TBE (0.089 mol/L tris-borate, 0.089 mol/L boric acid, and 0.008 mol/L EDTA) as running buffer.

### Table 2. Number of CFU-E-Derived Colonies/100,000 Cells Plated (average of 3 clots ± SD)

<table>
<thead>
<tr>
<th>Hours of epo Deprivation</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>15</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>157 ± 37.8</td>
<td>127</td>
<td>118 ± 3.5</td>
<td>98 ± 12.6</td>
<td>100 ± 26.4</td>
<td>40 ± 25.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>465 ± 138.6</td>
<td>480 ± 75.0</td>
<td>342 ± 24.7</td>
<td>185 ± 52.0</td>
<td>32 ± 7.6</td>
<td>29 ± 2.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>372 ± 136.6</td>
<td>313 ± 85.6</td>
<td>383 ± 47.5</td>
<td>222 ± 7.6</td>
<td>128 ± 36.2</td>
<td>60 ± 13.2</td>
<td>5 ± 0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>320 ± 31.2</td>
<td>146 ± 58.6</td>
<td>142 ± 93.0</td>
<td>113 ± 30.9</td>
<td>65 ± 18.0</td>
<td>60 ± 10.0</td>
<td>8 ± 2.9</td>
<td>3 ± 5.8</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>328.5 ± 111.8</td>
<td>266.5 ± 142.9</td>
<td>246.2 ± 117.4</td>
<td>154.5 ± 51.0</td>
<td>81.2 ± 36.1</td>
<td>40.5 ± 23.7</td>
<td>3.2 ± 3.4</td>
<td>0.8 ± 1.3</td>
</tr>
</tbody>
</table>

### Table 3. No. of BFU-E-Derived Colonies/100,000 Cells Plated (average of 3 clots ± SD)

<table>
<thead>
<tr>
<th>Hours of epo Deprivation</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>15</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>207 ± 83.7</td>
<td>175 ± 52.2</td>
<td>193 ± 7.6</td>
<td>115 ± 87.6</td>
<td>62 ± 24.7</td>
<td>23 ± 20.2</td>
<td>30 ± 15.0</td>
<td>8 ± 2.7</td>
</tr>
<tr>
<td>2</td>
<td>267 ± 88.1</td>
<td>283 ± 22.5</td>
<td>323 ± 84.6</td>
<td>390 ± 91.9</td>
<td>113 ± 29.3</td>
<td>35 ± 25.0</td>
<td>18 ± 7.6</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>342 ± 20.2</td>
<td>256 ± 106.1</td>
<td>228 ± 24.7</td>
<td>133 ± 63.5</td>
<td>33 ± 12.6</td>
<td>38 ± 36.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>128 ± 63.7</td>
<td>117 ± 14.4</td>
<td>85 ± 31.2</td>
<td>76 ± 25.0</td>
<td>37 ± 16.1</td>
<td>13 ± 7.6</td>
<td>12 ± 5.8</td>
<td>10 ± 1.4</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>236 ± 78.6</td>
<td>208 ± 65.8</td>
<td>207 ± 85.1</td>
<td>178 ± 124.4</td>
<td>61 ± 31.9</td>
<td>27 ± 10.0</td>
<td>15 ± 10.8</td>
<td>4.5 ± 4.6</td>
</tr>
</tbody>
</table>

### Liquid suspension culture

Cells (5 to 10 × 10^6) were placed in RPMI 1640 (GIBCO, Grand Island, NY) containing 20% (vol/vol) heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 10% (vol/vol) bovine serum albumin (Sigma), 10% (vol/vol) asparagine (0.2 mg/mL) (Sigma) and 20% (vol/vol) NCTC-109 (Whittaker Bioproducts, Walkersville, MD) at a concentration of 1 × 10^5 cells/mL. Recombinant human epo (Amgen) was added at a concentration of 2 U/mL to each flask according to a preset timetable, thus causing epo deprivation from 0 to 72 hours. The cells were harvested after 2 to 7 days in culture. Cell viability was confirmed by trypan blue exclusion.
5'-End DNA labeling. Five micrograms of total DNA was labeled with γ-32P-adenosine triphosphate (32P-ATP) (3,000 Ci/mmol; New England Nuclear [NEN], Boston, MA) as described in the instructions for the Boehringer-Mannheim 5'-end labeling kit (Boehringer-Mannheim Biochemical Co, Indianapolis, IN).

Southern blot analysis. Blotting on to Genescreen Plus transfer hybridization filter (NEN Research Products, Boston, MA) was performed as described in the instructions, using random 32P-nucleotide (α-32P-dATP, dCTP, dTTP; 3,000 Ci/mmol; NEN) labeled human DNA probe as described in the Boehringer-Mannheim Random Nucleotide Labeling Kit (Boehringer-Mannheim Biochemical Co, Indianapolis, IN). Densitometry of the autoradiogram was performed on an LKB Ultrascan Densitometer (LKB, Bromma, Sweden).

RESULTS

Colony expression as a function of epo deprivation. When CFU-E-derived colonies were enumerated as a function of the duration of epo deprivation in semisolid plasma clot culture there was a 50% loss of colony-forming capability (t1/2max) at approximately 3 hours in two DBA patients (LV and YD) compared with a mean of 9 hours for the four controls (Fig 1A). A total loss of CFU-E colony expression was noted at 9 hours in the DBA patients, whereas it required 48 hours of epo deprivation for total colony loss in the controls. CFU-E-derived colony counts are enumerated in Table 2. The BFU-E-derived colony response to epo deprivation displayed a similar pattern of decrement (Fig 1B). However, there was a greater shift in the t1/2 max from less than 3 hours for the two DBA patients to approximately 12 hours for the controls. In addition, a small number of BFU-E-derived colonies from controls more resistant to epo deprivation were noted. These more resistant colonies most likely represent the most immature, less epo-dependent BFU-E. This pattern is consistent with previous studies of hematologic normals.23 BFU-E-derived colony counts are enumerated in Table 3.

DNA fragmentation as a function of epo deprivation. The time course for DNA fragmentation as a function of epo deprivation was noted to be accelerated in DBA patients as evaluated by PAGE. DNA fragmentation observed at 0 hours of epo deprivation in DBA patients was in excess of that seen in controls at 0 hours, and was shown to increase by 3 hours of epo deprivation, whereas no significant increase in low molecular-weight DNA was apparent in the control until 9 to 15 hours of epo deprivation. A representative study, patient YD versus control, is shown in Fig 2. Total DNA degradation probably accounts for the decrease in low molecular DNA observed at 24 hours in the DBA patient. The presence of DNA fragmentation at 0 hours of epo deprivation most likely represents physiologic apoptosis and/or apoptosis occurring during processing of the marrow before culture.

Because of the heterogenous nature of BM MNCs, the ethidium bromide stain did not show the distinct DNA ladder indicative of apoptosis when homogenous cell populations or cell lines are evaluated. To show that the appearance of this low molecular-weight DNA was a consequence of apoptosis, rather than nonspecific degradation 5'-end DNA labeling was used to increase the signal of these small fragments. Figure 3 shows a similar time course as in Fig 2 for DBA patient JK with 5 μg of total DNA labeled per sample. Distinct oligosomes of 180 and 360 bp were noted at 3 hours in the DBA patient. Densitometry of the autoradiograph in Fig 4 quantitates the appearance of these distinct DNA oligosomes by 3 hours of epo deprivation. Typical oligosomes of 180 and 360 bp seen in DBA patients by 3 hours were not identified in normal controls until 9 to 15 hours of epo deprivation (data not shown). Comparable oligosome formation was again confirmed with this technique in 3 additional patients (PM, JB, and OT) in studies using fewer epo delay time points (Fig 5), showing that the low molecular-weight DNA resulting from epo deprivation is in the form of typical oligosomes characteristic of apoptosis.

DISCUSSION

Erythroid progenitors from patients with DBA were noted to have increased susceptibility to apoptosis as shown by the accelerated loss of clonogenicity in semisolid culture and an accelerated increase in characteristic DNA fragmentation when erythroid cells are deprived of epo. This finding is consistent with the proposed intrinsic erythroid-progenitor defect in which an abnormality in hematopoietic...
growth-factor responsiveness leads to the observed erythroblast failure in DBA. Indeed apoptosis as a mechanism of cell death has been described for other hematopoietic cells when appropriate growth factors (eg, granulocyte-macrophage colony-stimulating factor, IL-3) have been withheld, suggesting a common mechanism of proliferation control in which hematopoietic cells are programmed to die in the absence of required growth factors. In DBA, where the erythroid progenitors are relatively insensitive to epo, either as the primary defect or as a consequence of insensitivity to other growth factors, apoptosis may predominate over epo-induced proliferation and differentiation resulting in a decreased number and/or proliferative capacity of erythroid progenitors and precursors.

The pathways that lead to apoptosis are not completely understood and may differ in various cell types. The accelerated apoptosis noted in DBA may result from defective epo receptor-ligand interactions, abnormal intracellular signal transduction or even the erroneous initiation of apoptosis. A detailed understanding of receptor-epo interactions and the intracellular signaling events that follow recep-

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**Fig 3.** 5' End labeling of DNA. Five micrograms of total DNA from patient JK was labeled by 32P 5' end DNA labeling technique and analyzed by 7 mol/L urea, 8% PAGE. Lanes 1 through 7 correspond to 0, 1, 3, 6, 7.5, 9, and 15.5 hours of epo deprivation, respectively. By this technique, distinct DNA fragments were visualized, occurring at 3 hours of epo deprivation in the DBA patient.

**Fig 4.** Densitometry of 5' end labeled DNA fragments from 7 mol/L urea, 8% PAGE autoradiogram. The autoradiogram shown in Fig 3 was scanned across 180 bp and 360 bp regions. Distinct oligosomes were identified by 3 hours of epo deprivation.

**Fig 5.** 5'-End labeling of DNA. Five micrograms of total DNA from patients PM, (0, 3, and 8 hours, lanes 1 through 3), JB, (0, 7, and 25 hours, lanes 4 through 6) and OT, (0, 4, 5, and 8 hours, lanes 7 through 9) as in Fig 3. Distinct DNA oligosomes characteristic of apoptosis were visualized in all three DBA patients, beginning for the larger fragments, at zero hours. Right-hand arrows from top to bottom denote 720-, 540-, 360-, and 180-bp DNA fragments.
tor-ligand binding of all essential erythropoietic growth fac-
tors, as well as a precise delineation of the events leading to
the initiation of apoptosis will be required in order to dissect
the specific defects in DBA.

Recently, differences in the cytoplasmic region of the
human epo receptor have been proposed to account for the
differences in epo responsiveness of mature versus immu-
nute erythroid progenitors. 10 Whereas a truncated epo re-
ceptor predominates in immature erythroid progenitors, a
full length receptor is the most prevalent form in more ma-
ture progenitors. The full length receptor appears to trans-
duce a signal preventing programmed cell death. A defect in
this or a similar cytoplasmic region of the epo or another
receptor respectively in DBA could account for the acceler-
ated apoptosis observed in the erythroid progenitors from
these patients.

Although the DBA patients studied were representative of
the heterogeneity of this syndrome in that three were ini-
tially steroid responsive but became refractory, one steroid
resistant, two steroid responsive, and one responsive to IL-
3 at the time of study, additional patients will need to be
evaluated.

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