Eosinophil Peroxidase Deficiency: Morphological and Immunocytochemical Studies of the Eosinophil-Specific Granules

By Giuliano Zabucchi, Maria Rosa Soranzo, Renzo Menegazzi, Monica Vecchio, Alessandra Knowles, Clara Piccinini, Paola Spessotto, and Pierluigi Patriarca

Five eosinophil peroxidase (EPO)-deficient subjects were identified from 131,000 peripheral blood samples examined for routine automated analysis. The EPO-deficient eosinophils of these subjects met the main criteria established for EPO deficiency: absent or strongly decreased reaction for peroxidase, absent or strongly decreased staining with Sudan Black, and an increased ratio of the granule core volume to the total granule volume. In this report we show that this granule alteration is caused mainly by a decrease of its volume, particularly of the matrix, and that two other matrix proteins, eosinophil cationic protein and eosinophil derived neurotoxin, appear to be present in normal amounts in the EPO-deficient granules.

THE DEFICIENCY of eosinophil peroxidase (EPO) is a rare inherited anomaly, without evidence of disease, described for the first time by Presentey in 1968. The so-called Presentey’s anomaly has been characterized by cytochemical means at the optical level and, using these techniques, the defect was studied among the families of two probands. The ultrastructural analysis of the EPO-deficient eosinophil (EPOdE) has shown a remarkable alteration of their specific granules. In particular, the ratio between the core and the matrix size appears to be increased.

Although this alteration of the specific granules has been reported in EPOdE of all the EPO-deficient subjects examined until now, it has not been characterized at the ultrastructural level. Particularly, it is not known if the increase of the core-matrix ratio results from an enlargement of the crystal core or a decrease of the whole granule volume and whether or not a deficiency of granule proteins, other than EPO, is associated with the altered granule structure.

With the aim to discriminate among these possibilities we undertook an ultrastructural study on eosinophils obtained from five patients carrying the Presentey’s anomaly.

In this report we show that the defect of the granule is caused mainly by a decrease of its volume, particularly of the matrix, and that two other granule proteins, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), appear to be present in normal amounts in the granules of EPOdE, as judged by a postembedding immunogold cytochemical analysis.

MATERIALS AND METHODS

EPO-Deficient Subjects

Five cases of EPO deficiency were found among 131,000 peripheral blood routine tests performed at the Hematology Section of the Laboratory for Clinical Research of the Hospital Maggiore of Trieste from November 1988 to February 1992.

Of the five subjects identified, 1 and 3 belong to a group of hypercholesterolemic patients, subject 2 was a healthy blood donor, subject 4 suffered from depressive syndrome, and subject 5 did not take any drug. Subject 5 ended his chemotherapy and radiotherapy treatment caused mainly by a decrease of its volume, particularly of the matrix, and that two other granule proteins, eosinophil cationic protein and eosinophil derived neurotoxin, appear to be present in normal amounts in the EPO-deficient granules.

Cell Isolation

Peripheral blood, withdrawn with informed consent, from both normal and EPO-deficient subjects was collected in ACD solution (Don Baxter Laboratories, Trieste, Italy) and processed as previously described.

Briefly, the red blood cells (RBCs) were removed by dextran (0.75% wt/vol final concentration) sedimentation and the polymorphonuclear leukocytes were obtained by centrifuging the postdextran white blood cell (WBC)-rich plasma for 20 minutes at 250g. Granulocytes (neutrophil-eosinophil mixed populations) were separated from mononuclear cells by centrifuging the postdextran WBC-rich plasma for 20 minutes at 1,000g on isotonic Percoll (density 20°C = 1.077 g/mL). A 90-second hypotonic treatment was used to remove residual erythrocytes from the granulocyte-rich pellets. The WBCs were then centrifuged, washed once in Krebs Ringer phosphate (KRP) suspended in the same medium, and counted electronically (Coulter Counter ZBI, Luton, UK).

Light Microscopy

Cell suspensions were diluted to 0.2 10^6 cells/mL with KRP and deposited on glass slides with the use of a cytocentrifuge (Cytospin 2, Shandon, UK). The cells were stained for peroxidase according to Kaplow et al using 3,3′-diaminobenzidene tetrahydrochloride (DAB) as substrate, and with Sudan Black for phospholipids according to the method of Lison. Differential counts and quantitative evaluation of nuclear segmentation and the granule...
Table 1. Characterization of EPO-Deficient Eosinophils According to Presentey Criteria

<table>
<thead>
<tr>
<th></th>
<th>Sex</th>
<th>Age</th>
<th>Peroxidase Reaction</th>
<th>Sudan Black Staining</th>
<th>Nuclear Segmentation</th>
<th>No. of Granules</th>
<th>HVA Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal subjects</strong></td>
<td></td>
<td></td>
<td>Strong</td>
<td>Strong</td>
<td>13-17*</td>
<td>100†</td>
<td>169.1 ± 15.8†</td>
</tr>
<tr>
<td><strong>EPO-deficient subjects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>M</td>
<td>53</td>
<td>Absent/trace</td>
<td>Absent</td>
<td>39</td>
<td>48 52</td>
<td>ND</td>
</tr>
<tr>
<td>Subject 2</td>
<td>M</td>
<td>47</td>
<td>Absent/trace</td>
<td>Absent</td>
<td>30</td>
<td>100 —</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Subject 3</td>
<td>F</td>
<td>58</td>
<td>Trace</td>
<td>Trace</td>
<td>9</td>
<td>71 29</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Subject 4</td>
<td>F</td>
<td>65</td>
<td>Absent/trace</td>
<td>Absent/trace</td>
<td>50</td>
<td>33 67</td>
<td>ND</td>
</tr>
<tr>
<td>Subject 5</td>
<td>M</td>
<td>34</td>
<td>Absent</td>
<td>Absent</td>
<td>10</td>
<td>58 42</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Mother of subject 5</td>
<td>F</td>
<td>64</td>
<td>Intermediate</td>
<td>ND</td>
<td>ND</td>
<td>ND 78.0</td>
<td></td>
</tr>
</tbody>
</table>

Four normal subjects were studied. The EPO-deficient subjects were followed-up for a period ranging from 24 to 31 months. The values reported, obtained on the last observation, were virtually unchanged over the period of the study.

*The values reported for nuclear segmentation represent the percentage of eosinophils with more than two nuclear lobes.
†The values of granule number represent the percentage of eosinophils with normal or few granules per cell.
‡The values of HVA oxidation, reported from ref 11, are expressed as fluorescence units.

Number in eosinophils were performed on Wright- and Giemsa-stained specimens. Quantitative evaluation of peroxidase-positive cells and Sudan Black-positive cells was performed by scoring 50 to 100 eosinophils, and the intensity of the reaction graded as: strong, intermediate, trace, and absent. Micrographs were taken with an Orthoplan Leitz microscope (Wetzlar, Germany).

**Antisera**

Rabbit antisera against eosinophil peroxidase, eosinophil cationic protein, and eosinophil-derived neurotoxin were a kind gift of Prof G. Gleich (Mayo Clinic, Rochester, MN). ECP and EDN did not show cross-reactivity with their respective antibodies. A nonrelevant hyperimmune polyclonal rabbit antiserum against a mouse macrophage-specific antigen was a kind gift of Prof P. Dri (Istituto Di Patologia Generale, Università Di Trieste, Trieste, Italy), while the antihuman serum albumin rabbit antiserum was purchased from Behring (Seoppito, Italy).

**Electron Microscopy**

**Peroxidase cytochemistry.** Leukocytes were fixed in 2% glutaraldehyde (Serva, Heidelberg, Germany) and diluted in 0.1 mol/L cacodylate buffer pH 7.4 for 20 minutes at 4°C. For peroxidase staining, the cells were rinsed twice with 0.05 mol/L Tris-HCl pH 7.5 at room temperature, incubated in the same buffer containing 0.05% DAB and 0.02% H2O2 (Serva) freshly prepared for 60 minutes at room temperature, and rinsed with 0.1 mol/L cacodylate buffer pH 7.4 for 10 minutes. The cells were postfixed in 1% OsO4 for 60 minutes at 4°C, dehydrated in graded ethanols, and finally embedded in Dow Epoxy Resin (DER 332, Unione Chimica Europea, Milano, Italy and DER 732, Serva, Heidelberg, Germany). Ultrathin sections were cut by an ultrathome (Pharmacia-LKB, Uppsala, Sweden). These sections were stained with lead citrate.

**Immunostaining.** The immunogold postembedding technique, used to show EPO, ECP, and EDN on eosinophil sections, was performed on ultrathin sections stained with peroxidase.
performed essentially as previously described for eosinophils\textsuperscript{15,16} with minor modifications. Briefly, leukocytes, fixed as described above, were rinsed once, with 0.05 mol/L Tris-HCl and twice with 0.1 mol/L cacodylate buffer pH 7.4 for 10 minutes. The cells were postfixed in 1% OsO\textsubscript{4} for 60 minutes at 4°C, dehydrated in ethanol, and embedded as described above. Ultrathin sections were mounted on nickel grids, etched for 1 minute with 1% periodic acid, and rinsed in distilled water three times for 2 minutes each. The sections were then incubated in 20 mmol/L Tris-HCl pH 8.2 containing 1% bovine serum albumin (BSA) for 5 minutes at room temperature and exposed overnight at 4°C to the anti-EPO, anti-ECP, anti-EDN antisera, normal rabbit preimmune serum or nonrelevant polyclonal rabbit antisera diluted 1:100 in 20 mmol/L Tris-HCl pH 8.2 containing 1% BSA. The grids were washed three times (over 15 minutes) in the same buffer and were incubated thereafter with gold-labeled protein A (1:50 final dilution) for 1 hour at room temperature. The grids were finally rinsed five times (over 25 minutes) with buffer and distilled water (last washing) and double stained with uranyl acetate and lead citrate for 5 minutes each.

Quantitative evaluation of the antigen recognized by the antisera was performed by counting the gold particles in the cytoplasm, in the nucleus, and in the granule sections. The values were referred to the surface unit (cm\textsuperscript{2} of the micrograph) of the specific site, and reported as gold particle density. The surface area of the nucleus and cytoplasm were calculated by weighing their cut shapes traced on glossy paper. The total surface occupied by the granule in the cell section was calculated by adding up the area of each granule evaluated by the best fitting of their shape with a circumference of known radius. Because the gold particle density on nuclear sections was virtually the same in the presence of both preimmune rabbit serum and specific antisera, it was taken as the background value.

\textit{Morphometry.} Morphometric analysis was performed only on eosinophils showing the nucleus in the plane of the section. The mean volumes of the granule and core, relative to the cytoplasm volume (mean relative volume), were determined with point counting grids according to Weibel and Elias.\textsuperscript{17} The Student's \textit{t}-test for unpaired data was used to distinguish statistically significant differences.

\textit{Biochemical Assay of EPO Activity}

The method of Guibault et al.,\textsuperscript{18} based on the oxidation of homovanillic acid (HVA), as modified by Menegazzi et al.,\textsuperscript{11} was used to measure EPO activity in eosinophil-neutrophil mixtures.

\textit{Reagents}\n
Dextran (Dextran T-500) and Percoll were obtained from Pharmacia (Uppsala, Sweden); DAB was purchased from Serva (Heidelberg, Germany); Sudan Black was supplied by Sigma Co (St Louis, MO). Other chemicals were of reagent grade. KRP contained 122 mmol/L sodium chloride, 4.9 mmol/L potassium chloride, 1.2 mmol/L magnesium chloride, 0.5 mmol/L calcium chloride, 16 mmol/L sodium phosphate buffer pH 7.4, 5 mmol/L glucose, and 0.5% (wt/vol) BSA (Miles Italiana S.p.A., Milano, Italy). Gold-labeled (20 nm) protein A was purchased from E.Y. Laboratories Inc (San Mateo, CA).

\textbf{RESULTS}

\textit{Identification and Characterization of the Eosinophil-Peroxidase-Deficient Subjects}

From 131,000 peripheral blood samples examined for routine analysis by automated flow cytochemistry in the Hospital Maggiore of Trieste from November 1988 to February 1992, five subjects were identified whose cytoc-grams showed an empty eosinophil box. Eosinophils were present in the Wright-Giemsa–stained peripheral blood smears of these subjects in a percentage ranging from 2.0 to 8.7 of the total WBCs. Because eosinophils are classified on
the basis of EPO reaction in the automated flow cytochemistry technique used, the five subjects were considered as potentially EPO deficient and were selected for confirmatory tests to be performed. The mother of one of them was also available for study.

Table 1 and Figs 1 through 3 show that all the five subjects met the main criteria established for EPO deficiency: (1) absent or strongly decreased cytochemical reaction for EPO (Figs 1a through c and 2); (2) absent or strongly decreased eosinophil staining with Sudan Black (Fig 1d through f); (3) absent or decreased biochemically detectable EPO activity (Table 1); (4) increased ratio of the core volume to the total eosinophil granule volume (Fig 2). Nuclear hypersegmentation and a decreased number of granules were also detectable in some of our subjects (Table 1 and Fig 3), but these anomalies were neither prominent nor frequent.

The five subjects listed in Table 1 were not consanguineous. Three of them (subjects 2, 3, and 5) have been reported by us previously. Earlier studies have shown the inherited nature of the defect in subject 5, his mother being a probable heterozygote for the EPO defect (Table 1). Direct evidence for a primary nature of the defect in the other subjects is not available.

Morphometric Analysis

A morphometric study of the granules of the eosinophils of subjects 1, 2, 3, and 5 was performed and the results are reported in Table 2. The mean relative volumes of the whole granules was smaller in all of the EPO-deficient eosinophils than in normal counterparts. In the eosinophils of subjects 1, 2, and 5, the relative volume of the core was also smaller than in normal cells. The decrease of the volume of the whole granule was always greater than that of the core, resulting in a marked decrease of the matrix volume and in an increased core-matrix ratio.

The altered granules were present in all the cells of subjects 1 through 5 and in 42% of the eosinophils of the mother of subject 5. On the contrary, granules with an altered morphology were rarely seen in normal eosinophils (<5%). Among the entire granule population of each cell section, a large percentage (ranging from 40% to 50%) of granules in EPO-deficient subjects, no more than 15% of the granules in the mother of subject 5 and virtually no granule in normal cells were shown to be altered.

Detection of EPO, ECP, and EDN by Immunogold Reaction

Figure 4 shows the results of the electron microscopic immunocytochemical studies on the normal and EPO-deficient eosinophils. Using an anti EPO polyclonal antiserum obtained from rabbits immunized with purified EPO, numerous gold particles were seen in the matrix of normal eosinophil granules while no or very few particles were observed in the granules of EPOdE.

In parallel with EPO detection, immunocytochemistry of two other granule proteins, ECP and EDN, was also performed by the same technique. There were no apparent differences in the number of gold particles present in the matrix of the granules of normal and EPO-deficient eosinophils with either the anti-EDN or the anti-ECP antisera. Figure 5 shows the morphologic appearance of the immunogold reaction for ECP in EPOdE (subject 5) as compared with a normal eosinophil (inset in Fig 5).

The immunogold reaction, which was shown to give quantitative information on the amount of antigen recognized, was quantified by enumerating the gold particles on eosinophil sections showing two nuclear lobes. The results reported in Table 3 show absence (subjects 4 and 5) or a strong decrease (subjects 1, 2, and 3) of antigenically reactive EPO. Instead the number of gold particles observed in EPOdE with the anti-ECP and the anti-EDN antisera did not significantly differ from the normal values,

### Table 2. Morphometric Analysis of Eosinophil Granules

<table>
<thead>
<tr>
<th></th>
<th>Mean Relative Volume $\times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Granule</td>
</tr>
<tr>
<td>Normal subjects</td>
<td>108.2 ± 0.7</td>
</tr>
<tr>
<td>EPO-deficient subjects</td>
<td></td>
</tr>
<tr>
<td>Subject 1</td>
<td>82.6 ± 1.3*</td>
</tr>
<tr>
<td>Subject 2</td>
<td>64.6 ± 0.4*</td>
</tr>
<tr>
<td>Subject 3</td>
<td>83.3 ± 1.9†</td>
</tr>
<tr>
<td>Subject 5</td>
<td>68.9 ± 0.5*</td>
</tr>
</tbody>
</table>

*P values were calculated from Student's t-test (two-tailed) on unpaired data (EPO-deficient subjects vs normal subjects). Values are expressed as means ± SEM. The number of granules scored ranged from 300 to 1,020.

*P < .001.
†P < .05.
‡Not significant.
indicating that the content of ECP and EDN is normal in EPOdE granules. As previously reported, EPO, ECP, and EDN are mainly localized in the granule matrix. Accordingly, the results reported in Table 4 show that in the cytoplasm of control eosinophils few gold particles were seen when anti-EPO, anti-ECP, and anti-EDN antisera were used, while the number of gold particles, showing ECP and EDN in the cytoplasm of EPOdE, was more than twice that in the cytoplasm of normal cells. Figures 5 and 6 show that these cytoplasmic particles were mainly localized in and around the area occupied by the granules. In the cytoplasm the gold particles were often set in a row, as in a
A necklace, on filamentous structures connecting the granules to each other (Fig 6). The same features were observed where the anti-EDN antiserum was used (not shown).

When a preimmune serum was used, instead of an anti-EPO, anti-ECP, or anti-EDN serum, virtually no gold particles were observed in any subcellular site (a value of average gold-particle density < 1 particle/cm² was obtained). The specificity of the immunogold reaction was further proven in both normal subjects and EPO-deficient subjects by using in the immunostaining procedure two different, nonrelevant, rabbit polyclonal antisera (see Materials and Methods). In these experimental conditions, the gold-particle density was equal to or even less than (Fig 7) that observed with preimmune rabbit serum.

**DISCUSSION**

We show here that the increased core-granule ratio that characterizes the EPOdE is accounted for by a decreased volume of both the matrix and the core, the former component being more prominently affected than the latter. Hence, the volume of the whole EPOdE granule is decreased and the organelle is mostly occupied by the core.

**Table 3. Quantitative Evaluation of Immunogold Particles in Eosinophil Granules**

<table>
<thead>
<tr>
<th>EPO</th>
<th>ECP</th>
<th>EDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>37.6 ± 3.7 (241)</td>
<td>59.3 ± 9.6 (277)</td>
</tr>
<tr>
<td>Subject 1</td>
<td>1.8 ± 0.4 (153)</td>
<td>70.0 ± 10.5 (115)</td>
</tr>
<tr>
<td>Subject 2</td>
<td>1.0 ± 0.3 (120)</td>
<td>65.7 ± 5.0 (125)</td>
</tr>
<tr>
<td>Subject 3</td>
<td>2.4 ± 0.4 (109)</td>
<td>67.1 ± 11.8 (112)</td>
</tr>
<tr>
<td>Subject 4</td>
<td>0.0 (100)</td>
<td>40.2 ± 6.0 (130)</td>
</tr>
<tr>
<td>Subject 5</td>
<td>0.3 ± 0.2 (192)</td>
<td>53.0 ± 10.7 (105)</td>
</tr>
</tbody>
</table>

Values represent the mean number of gold particles/cm² ± SEM counted on the number of granule sections reported in parentheses. Two normal subjects were studied. The background value, calculated by counting the gold particles within the nuclear area, has been subtracted. The background varied from 1.2 to 2.1 gold particles/cm² for EPO, from 0.7 to 2.0 for ECP, and from 0.7 to 2.3 for EDN. The density of gold particles is referred to the unit surface of granule sections. When preimmune rabbit serum substituted for anti-eosinophil protein sera in the incubation medium, the number of gold particles/cm² was always < 0.7.

Abbreviation: ND, not done.

**Table 4. Quantitative Evaluation of Immunogold Particles in Eosinophil Cytoplasm**

<table>
<thead>
<tr>
<th>EPO</th>
<th>ECP</th>
<th>EDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>4.1 ± 0.7</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td>Subject 1</td>
<td>3.9 ± 1.8</td>
<td>20.5 ± 6.3</td>
</tr>
<tr>
<td>Subject 2</td>
<td>2.4 ± 0.6</td>
<td>19.7 ± 4.6</td>
</tr>
<tr>
<td>Subject 3</td>
<td>3.3 ± 0.8</td>
<td>25.0 ± 10.4</td>
</tr>
<tr>
<td>Subject 4</td>
<td>0.0</td>
<td>10.8 ± 0.7</td>
</tr>
<tr>
<td>Subject 5</td>
<td>1.2 ± 0.6</td>
<td>16.0 ± 3.2</td>
</tr>
</tbody>
</table>

Values represent the mean number of gold particles/cm² ± SEM counted in the cytoplasm of 5 to 7 cell sections. For other details see Table 3.

Abbreviation: ND, not done.
Fig 6. Immunogold labeling of blood eosinophils with polyclonal anti-ECP antibodies. A part of an eosinophil from subject 3 is shown. The particles are found both in the granules and in the cytoplasm. The cytoplasmic gold particles were frequently aligned along filamentous structures (arrows). Original magnification × 41,000.

with the matrix appearing as a tiny rim around it. This may give the impression, when observing micrographs of EPOdE, of an increased core volume, which is not supported, however, by careful morphometric analysis such as the one performed in the present study.

It is important to point out that the anomaly observed was present in all the eosinophils of all five EPO-deficient subjects studied. The degree of granule abnormality varied from granule to granule in a single cell along a spectrum ranging from granules with a nearly normal matrix volume (very few) to granules with a very thin matrix shell. Instead the great majority of the granules of the eosinophils of the heterozygote subject were normal, no more than 15% of them showing an appreciable decrease of the matrix volume.

EPO deficiency is, at the moment, the sole documented protein deficiency in the Presentey’s anomaly. In fact the two other major cationic proteins of the matrix, i.e., ECP and EDN, were antigenically detectable in normal amounts, as shown in this report, and the activity of acid and alkaline phosphatase, adenosine triphosphatase, and two esterases have been found, by cytochemical means, to be normal in EPOdE by other investigators.7,10

However, the experiments of immunogold localization of ECP and EDN in EPOdE provided a peculiar feature. In fact, most of the gold particles were located in the matrix in both normal and EPOdE, but the number of cytoplasmic gold particles in EPOdE was about twice as high as in normal eosinophils. This suggests a defect in either the packing of these two proteins in the abnormal granule or a leakage from it.

The cytoplasmic gold particles in EPOdE were aligned along ill-defined filamentous structures whose significance is unknown at the moment.

Is the decreased amount of EPO in the granule matrix sufficient per se to explain the decreased matrix volume? The fact that EPO accounts for a large percentage of the granule protein (as calculated from the references cited in 20) would suggest a positive answer to the question. However, the virtually normal morphology of the granules of the eosinophils of the heterozygote subject, which contained about 45% of the normal EPO activity, leaves open the possibility that other factors may be involved in the altered granule morphology.

To the best of our knowledge, 108 EPO-deficient subjects have been described thus far in the literature.1-10 At least in the 12 cases in which a full family study could be performed, the defect has been shown to be of genetic origin2-4 and in 18 other cases a family link has been shown.1,6,21 Of the five subjects described here, subject 5 had a genetic defect because a previous study based on the biochemical assay of EPO in his and his relatives’ eosinophils showed an autosomal pattern of inheritance.11

We have no direct evidence concerning the nature of the EPO defect in the other four subjects described here because their families were not available for study. However, the fact that their defect remained qualitatively and quantitatively unchanged over a period of at least 24 months, during which three determinations were performed, suggests a possible genetic basis of the defect even in these cases.

The five EPO-deficient subjects described in the present study were identified among 131,000 subjects tested for automated cytochemical analysis of peripheral blood samples over a period of 40 months. Because the samples tested may include repeats on the same subjects, it is safe to conclude that the prevalence of the defect in the city of Trieste and its province is probably higher than 1:26,000.

None of our EPO-deficient subjects was of Yemenite, North African, or Iraqi Jewish origin. The first reported subjects with EPO deficiency and most of the other subjects with the defect described in the world literature thus far (90
of 108) belong to these ethnic groups, so that the suggestion has been put forward that the defect is not rare among these groups and may be characteristic of them. However, the cases described by other investigators together with the five subjects reported by us indicate that the defect is more geographically widespread than originally thought. Finally, it is worth noting that the recently achieved cloning of EPO provides the basis for an accurate molecular analysis of the EPO defect that is presently in progress in our laboratory.

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ACKNOWLEDGMENT

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