Clinical Features and Studies of Erythropoiesis in Israeli Bedouins With Congenital Dyserythropoietic Anemia Type I

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Congenital dyserythropoietic anemia (CDA) type I is a rare macrocytic anemia of unknown etiology. In the present study, we redefined the clinical and laboratory picture of CDA type I, some of its pathogenic aspects, and the association with thalassemias in 20 patients, all of whom belong to one Bedouin tribal group and are probably descended from a common ancestor. In each case ultrastructural studies of bone marrow (BM) erythroblasts showed the classic morphological findings of CDA type I. Serological tests for CDA type II were negative. The clinical picture was variable, but mostly benign. Some patients displayed elevated hemoglobin A2 levels or a high ratio of α-to non-α-globin. However, neither family studies nor complete sequence analysis of the β-globin was compatible with β-thalassemia. Increased erythropoiesis was manifested by a high number of BM erythroid burst-forming units. Serum erythropoietin was also elevated. BM flow cytometry studies demonstrated arrest of erythroid precursors in the S phase of the cell cycle. The ultrastructural morphological features of the erythroid precursors, showing peripheral chromatin condensation, suggest apoptosis. Additional studies are indicated to define the molecular basis of this disease.

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CONGENITAL dyserythropoietic anemias (CDAs) are a rare group of red blood cell (RBC) disorders of unknown etiology characterized by ineffective erythropoiesis, pathognomonic cytopathology of the nucleated RBCs in the bone marrow (BM) and secondary hemochromatosis. In 1968, Heimpel and Wendl classified these disorders into three types, which were later confirmed by electron microscopy and serological findings. Type I is characterized by congenital macrocytic anemia, megaloblastoid erythroid hyperplasia, and the presence of nuclear chromatin bridges between erythroblasts. More than 60 sporadic cases of CDA type I have been reported in the literature. The typical morphological ultrastructural studies accompanied by negative acidified serum lysis and adult i/i agglutination test constitute the major diagnostic features. The more common type II, also known as hereditary erythroblast multinuclearity with a positive acidified serum test (HEMPAS), is characterized by normocytosis or macrocytosis with binucleated and multinucleated marrow erythroblasts and karyorrhexis. Type III is manifested by macrocytosis and erythroblastic multinuclearity with prominent gigantoblasts.

More than 30 documented cases could not be clearly assigned to type I, II, or III. Type IV was the classification used in four reports in which BM morphology resembling that in type II was accompanied by negative acid serum tests. CDA in association with thalassemia has been described in 17 patients. Members of more than a dozen other families had CDAs that were even more difficult to classify.

Anselstetter et al. in 1977 were the first to report that band 3 glycoproteins from HEMPAS erythocyte membranes migrate faster than those obtained from normal erythrocyte membranes. Reduced levels of N-glycans in HEMPAS erythocyte membranes with low N-acetylglycosaminyltransferase II activity have been documented in some patients, and a defect in the α-mannosidase II gene was reported in one patient. Faulty glycosylation of transferrin, a glycoprotein produced in the liver, has also been described. The molecular basis for the defects in other CDAs is not known.

In the following study, we describe 20 patients with CDA type I who belong to seven Bedouin families living in the Negev (the southern part of Israel). The aim of the study was to redefine the clinical and laboratory characteristics of CDA type I in this homogeneous population in an attempt to clarify the pathogenesis of the disorder and its association with thalassemia.

MATERIALS AND METHODS

Twenty patients (14 men, 6 women), aged 1 to 23 years (median, 10 years) from seven families were evaluated. Three patients were originally diagnosed and followed at our center, and the medical records of the 11 patients followed for anemia at the Soroka Medical Center in Beersheva were reviewed. Six additional patients were identified in the course of family studies. Each of the 20 patients underwent a complete physical examination. Bone age was determined in six patients. Control BM samples for culture studies and flow cytometry studies were obtained from healthy children who were donors for BM transplant. β-thalassemia major BM samples were obtained at diagnosis. The following blood and BM examinations were performed for each patient.

Routine blood tests. After obtaining informed consent, venous blood was drawn. A complete blood count was performed using an H1 counter (Technicon, Israel). Measurement of serum folic acid and vitamin B12; levels, hemoglobin electrophoresis, determination of anti-I and i antigen titers, and Ham and Coombs tests were all performed using standard techniques. Ferritin levels were measured by a sandwich enzyme immunoassay (Melisa Kit, Cambridge Life Science, Cambridge, England). Serum erythropoietin (EPO) levels were determined using a diagnostic kit with microtiter wells.

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coated with antierthropoietin-specific IgG (JCL Clinical Research Corporation, Knoxville, TN).

**BM electron microscopic studies.** For ultrastructural evaluation, aspirated BM cells were fixed overnight at 4°C in Karnovsky's fixative in 0.1 mol/L cacodylate buffer and postfixed in 1% osmium tetroxide for 1 hour. After washing in cacodylate buffer, the specimens were subjected to dehydration by passage through a graded series of ethanol concentrations and propylene oxide and finally embedded in epon 812. Thin sections were then collected on copper grids, counterstained with uranyl acetate and lead citrate, and examined under a Philips 201 electron microscope. Peripheral erythroid colonies were treated as above and observed under an electron microscope.

**BM and peripheral blood culture studies.** BM and peripheral blood were obtained for assays of erythroid colony formation. Heparinized BM or peripheral blood were layered over a discontinuous Histopaque-1077 gradient (Sigma Diagnostics, St Louis, MO) and centrifuged (200g, 4°C) for 25 minutes to remove neutrophils and

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**Fig 1.** Electron microscopy of erythroid precursors in BM. (A) Four normoblasts with typical CDA type 1 features: sponge-like heterochromatin; enlargement of nuclear pores; invagination of cytoplasm into nuclear area (magnification × 4,766). (B) Left lower normoblast of (A): spongy heterochromatin structure (a); enlarged nuclear pore (b); invagination of cytoplasm into nucleus; iron-loaded mitochondria (arrow) (magnification × 24,126). (C) Typical binucleated CDA type I normoblast with nuclear bridge (magnification × 8,896). (D) CDA type I normoblast showing prominent apoptotic features: peripheral nuclear condensation of heterochromatin (arrows), disruption of nuclear pores (arrowhead) and conservation of cytoplasmic organelles. Mt, mitochondria, (magnification × 21,760).
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Fig 2. Pedigree of families with CDA type I. Blocked symbols represent subjects diagnosed as CDA type I; hatched symbols represent subjects with CDA type I but not available for study.

RBCs. The buoyant mononuclear cells obtained from the gradient interface were washed twice in Iscove’s modified Dulbecco medium. Erythroid colony assays were performed as previously described. Sensitivity to erythropoietin was determined by using increasing concentrations of EPO (1 to 8 U/mL). For assay of peripheral colonies, the cultures were supplemented with EPO (2 U/mL) and interleukin-3 (10 U/mL).

Cytogenetic studies. Chromosome analysis was performed on BM samples directly and after 24 hours culture and on peripheral blood lymphocytes stimulated for 2 hours with phytohemagglutinin. A trypsin-Giemsa technique was used for chromosomal banding. Chromosomes were described according to the International System for Human Cytogenetic Nomenclature.

Table 1. CDA Type I-β-Thalassemia-Like Features

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Hb (g/L)</th>
<th>MCV (fl)</th>
<th>HbA2 (%)</th>
<th>Hbf (%)</th>
<th>α/β + γ</th>
<th>β-Globin Gene Sequence</th>
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<tbody>
<tr>
<td>F1-1</td>
<td>73</td>
<td>93</td>
<td>3.9</td>
<td>1.1</td>
<td>1.28</td>
<td>N</td>
</tr>
<tr>
<td>F1-2</td>
<td>79</td>
<td>106</td>
<td>2.9</td>
<td>5.0</td>
<td>1.38</td>
<td>N</td>
</tr>
<tr>
<td>F1-3</td>
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<td>105</td>
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<td>1.1</td>
<td>1.39</td>
<td>N</td>
</tr>
<tr>
<td>F2-1</td>
<td>84</td>
<td>97</td>
<td>3.8</td>
<td>1.6</td>
<td>1.15</td>
<td>N</td>
</tr>
<tr>
<td>F2-2</td>
<td>93</td>
<td>104</td>
<td>3.4</td>
<td>5.8</td>
<td>1.25</td>
<td>N</td>
</tr>
<tr>
<td>F3-1</td>
<td>85</td>
<td>98</td>
<td>3.3</td>
<td>1.0</td>
<td>1.00</td>
<td>N</td>
</tr>
<tr>
<td>F3-2</td>
<td>94</td>
<td>95</td>
<td>3.4</td>
<td>1.1</td>
<td>0.97</td>
<td>N</td>
</tr>
<tr>
<td>F3-3</td>
<td>90</td>
<td>105</td>
<td>3.1</td>
<td>0.5</td>
<td>1.72</td>
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<tr>
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<td>N</td>
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<td>101</td>
<td>3.5</td>
<td>1.4</td>
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<tr>
<td>F4-4</td>
<td>81</td>
<td>102</td>
<td>3.3</td>
<td>1.2</td>
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<td>N</td>
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<tr>
<td>F7-1</td>
<td>84</td>
<td>97</td>
<td>3.0</td>
<td>6.0</td>
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<td>N</td>
</tr>
<tr>
<td>F7-2</td>
<td>82</td>
<td>92</td>
<td>3.9</td>
<td>1.6</td>
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<td>N</td>
</tr>
<tr>
<td>Mean</td>
<td>85</td>
<td>98</td>
<td>3.5</td>
<td>2.0</td>
<td>1.27</td>
<td>0.18</td>
</tr>
<tr>
<td>± SD</td>
<td>6</td>
<td>5</td>
<td>0.5</td>
<td>1.9</td>
<td>0.18</td>
<td></td>
</tr>
</tbody>
</table>

Normal

Mean 120 | 81 | 2.5 | <1 | 1.06
± SD 6 | 6 | 1 | 0.19

Abbreviation: N, normal sequence.
Fig 3. Marrow erythroid colonies dose-response curves to EPO. The number of CFU-E and BFU-E derived colonies/2 x 10^5 cells in response to EPO, 1 to 8 U/ml is depicted. Each point represents the mean result of six different experiments, the bars expressing the standard error of the mean (SEM). (A) control marrow; (B) CDA type I marrow showing increased numbers of BFU-E and CFU-E as compared with normal marrow.

Samples of whole marrow mononuclear cells and CD45-negative and -positive cells were stained with propidium iodide (PI) to determine DNA content. The fluorescence of the PI-stained cells was measured, as previously described, with a FACS-star analyzer. Cell cycle distribution was calculated according to the "Cell FIT" computer program. (Becton Dickinson, Immunofluorocytometry System, Mountain View, CA). Cell cycle distribution was calculated according to the "Cell FIT" computer program. (Becton Dickinson, Immunofluorocytometry System, Mountain View, CA). Samples of whole marrow mononuclear cells and CD45-negative and -positive cells were stained with propidium iodide (PI) to determine DNA content. The fluorescence of the PI-stained cells was measured, as previously described, with a FACS-star analyzer (Becton Dickinson, Immunofluorocytometry System, Mountain View, CA). Cell cycle distribution was calculated according to the "Cell FIT" computer program (version 2.1) (Becton Dickinson, San Jose, CA). Cell cycle distribution was calculated according to the "Cell FIT" computer program (version 2.1) (Becton Dickinson, San Jose, CA). Bromodeoxyuridine (BrdU) staining was performed as previously described. In brief, BM cells were pulse-labeled for 30 minutes with 10 μmol/L BrdU, washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin, fixed in 70% cold ethanol for 30 minutes on ice, and rehydrated overnight in PBS at 4°C. Staining with anti-BrdU antibodies conjugated to fluorescein isothiocyanate (Becton Dickinson) was performed after acid denaturation (incubation with 2N HC1-0.5% Triton X-100 for 30 minutes) and neutralization with NaZB407 (pH 8.5). The cells were subjected to RNase A before analysis. For double staining, 1 pg PVmL was used.

**Globin chain synthesis.** The globin synthesis ratio of peripheral blood reticulocytes was calculated after separation of globin chains labeled with 1H-leucine on a carboxymethyl cellulose column.

**β-Globin gene amplification and sequencing.** Genomic DNA was isolated from white blood cells, and the β-globin gene was amplified in two fragments by the polymerase chain reaction. The biotin-avidin system and magnetic beads were used to produce high-quality single-stranded template DNA, which was then sequenced directly by the dideoxy chain termination method.

**RESULTS**

**Diagnosis of CDA type I.** Diagnosis of CDA type I was based on the presence of macrocytic anemia and typical BM findings under light microscopy, including large erythroblasts with incomplete nuclear division and a chromatin bridge connecting the nuclei of two cells. Diagnosis was confirmed in all patients on the basis of typical BM electron microscopy characteristics, including spongy heterochromatin, enlargement of nuclear pores, invagination of the cytoplasm into the nuclear area, and iron-loaded mitochondria (Fig 1). Serological tests for CDA type II were all negative.

**Clinical data.** Fourteen of the 20 patients studied were the products of consanguineous marriages (Fig 2). In six families, there was more than one affected child. All families, although thought to be unrelated, belong to the same tribal group. Fourteen patients presented with anemia, while six were detected in the course of family studies. The age at diagnosis varied from birth to 23 years. Eight patients were diagnosed during the neonatal period, while an additional three were diagnosed between the age of 1 and 12 months.

Six patients required occasional blood transfusions. Two patients presenting at the neonatal period were more severely affected. One patient required monthly RBC transfusions until he underwent splenectomy at the age of 4 years and became transfusion-independent with Hb levels of 8 to 9.5 gr%. The other patient required RBC transfusions twice a year, however, because of increasing splenomegaly and transfusion requirement, splenectomy was performed at the age of 5 1/2 and since then she has maintained a hemoglobin level of 8.5 to 10.5 gr% with no need for transfusion.

**Growth parameters and physical examination.** The
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Fig 4. Electron microscopy of peripheral BFU-E. (A) Part of normal peripheral blood BFU-E colony at day 10 (magnification × 1,874). (B) Part of peripheral blood BFU-E colony from patient with CDA type I, displaying typical dyserythropoietic features (magnification × 5,504). (C) Erythroblast from above BFU-E colony showing spongy appearance of heterochromatin (a); enlarged nuclear pores (b); invagination of cytoplasm into nucleus (arrow); iron-loaded mitochondria (arrowheads) (magnification × 15,200).

height standard deviation score (SDS) was calculated and expressed as the number of standard deviations from the mean of each patient's height, as described by Tanner et al.31 The mean height SDS was -1.55 ± 1.02 with only four patients more than 2 SD below the mean height for their age. All patients presented with mild icterus, 11 had mild splenomegaly, and 10 had mild hepatomegaly.

Laboratory data. Hemoglobin levels ranged from 72 to 104 g/L (mean, 86 ± 8 g/L); mean corpuscular volume (MCV) was high, ranging from 90 to 107 fL (mean, 101 ± 5.3 fL). Absolute reticulocyte counts ranged between 18 to 112 × 10^12/L (mean, 51 ± 22 × 10^12/L). Indirect bilirubin, as well as serum lactate dehydrogenase, were elevated, haptoglobin was low, and ferritin was moderately elevated, ranging from 48 to 1245 µg/L (mean, 385 ± 293 µg/L; normal value, 10 to 300 µg/L). The results of the acidified serum test were negative and i/i titers were within the low adult range. Folic acid and B12 levels were normal. Serum EPO levels were mildly elevated: 18 to 112 miu/mL (mean, 53 ± 27 miu/mL; normal range, 22 to 54 miu/mL). Chromosomal studies of four patients showed a normal peripheral blood and BM karyotype. RBC membrane studies did not show any abnormality in the polypeptide membrane pattern or the abnormal, fast moving band 3 previously reported in CDA type II.7 No globin excess was present. The percentage of hemoglobin (Hb) A2 was abnormally high (range, 3.8% to 5.1%) in four of the 14 patients examined. The parents of the patients in family 1 (Fig 2) had normal counts and Hb A2 and Hb F levels. In nine of the 14 patients, globin synthetic ratios were abnormal (1.25 to 1.72), within the β-thalassemia minor range (Table I).
**BM and peripheral blood culture tests.** A 314% ± 141% increase in marrow colony forming unit-erythroid (CFU-E) was found upon the addition of erythropoietin (2 U/mL), with burst forming unit-erythroid (BFU-E) rising only to 164% ± 46%, and colony forming unit-culture (CFU-C) growth 162% ± 61% that of the control. There was a mild increase in CFU-E and BFU-E, with increasing erythropoietin concentrations (Fig 3). Serum supplementation was not associated with greater numbers of erythroid colonies. The number of BFU-E in peripheral blood cultures was 140% ± 86% that of the control. Ultrastructural BFU-E studies showed abnormal erythroid cell morphology, with a spongy appearance of the condensed chromatin and enlargement of nuclear membrane pores (Fig 4).

**BM DNA flow cytometry studies.** Normal and β-thalassemia major BM had normal DNA content, with most cells in the Go/G1 phase. However, in four of the patients with CDA type I, 47.6% ± 2.7% of the BM cells were in the S phase, versus 2.9% ± 1.7% of normal marrow cells (Fig 5). On separation of the erythroid cells, the pathological findings proved to be confined to the erythroid precursors, whereas the myeloid and lymphoid cells (CD45+) had a normal DNA content. Whole and separated BM cells were doubly stained with PI and fluorescent anti-BrdU (Fig 6). Double-parameter (BrdU plus PI) analysis distinguishes between cells engaged in active DNA synthesis and those arrested in the S phase. As can be seen in Fig 6, 35% of the CD45- mononuclear (erythroid) cells were arrested in the S phase.

**Globin synthesis and globin gene sequence.** Although four of the 14 patients examined displayed normal A2 values and six of the 14 displayed aberrant globin synthetic ratios (Table 1), base changes causing β-thalassemia were not evident on sequencing of the β-globin gene in four such patients. Furthermore, the parents of three CDA type I siblings with increased levels of hemoglobin A2 and abnormal globin synthetic ratios had normal hemoglobin, MCV and hemoglobin A2 values, and normal globin synthetic ratios (Fig 2, family 1), thus excluding any association with β-thalassemia.

**DISCUSSION**

About 60 cases of CDA type I have been reported in the literature, most of them sporadic. We studied 20 Israeli...
Bedouins with CDA type I (Fig 2). The Bedouins living in Israel originated in the Arab Peninsula and belong to three main tribal groups (confederations). Although all our patients are members of what seem to be unrelated families, they all belong to the same tribal group and probably stem from a common ancestor. The observed familial pattern clearly favors recessive autosomal inheritance as previously suggested. Increased erythropoiesis was manifested in our CDA type I patients by the large numbers of BM and peripheral blood erythroid colonies displaying the characteristic findings of condensed nuclear chromatin and abnormal nuclear membrane pores (Fig 3). The high number of peripheral blood and marrow CFU-E colonies, as well as their abnormal morphology, may suggest involvement of the committed stem cells and could serve as an additional diagnostic tool in CDA type I.

Variant globin chain synthesis has been described in 17 CDA patients from nine different families. Although some of these patients may represent authentic cases of thalassemia with coexistent CDA, none was documented definitively. Our β-globin sequence data on four patients, augmented by family studies (Fig 2), suggest that in our CDA type I patients, β-thalassemia can be excluded and that the imbalance in globin chain synthesis is probably secondary to the basic erythropoietic defect.

Several pathogenic mechanisms have been implicated in CDA type I. The plasma membrane has been considered a possible site of the primary lesion, with nuclear membrane breakdown secondary to the abnormal plasma membrane. We did not find any abnormal band on RBC membrane protein electrophoresis. Theoretically, nuclear membrane defects and a sponge-like nucleus could also be caused by abnormal histones. Indeed, abnormal histone content has been reported in CDA type I erythroblasts. Heimpel and Wndt, and later Wickramasinghe and Pippard, using Feulgen microspectrophotometry and [3H]-thymidine autoradiography, demonstrated gross abnormalities of proliferation in early polychromatophilic erythroblasts. The aberrations included arrest of DNA synthesis after cell progression through part of the S phase and formation of mononucleate and binucleate cells with hypertetraploid DNA content.

Based on earlier findings, we performed DNA flow cytometry studies on BM from four CDA type I patients (Fig 5). An increased number of erythroid cells in the S phase of mitosis were found in all of them. DNA content and cell cycle distribution were normal in BM CD45 + (nerythro) cells (Fig 5C) and in normal and β-thalassemia major whole BM (Fig 5A). Flow cytometry studies and cell cycle analysis, using double labeling with PI and BrdU (Fig 6), showed the arrest of CDA type I marrow erythroid cells in the S phase.

Apoptosis, programmed cell death, involves in series of morphological changes. Nuclear changes in early stages of apoptosis begin subjacent to the nuclear membrane. The chromatin assumes a characteristic pattern, extending as a band beneath the inner lamina of the nuclear membrane. The peripheral condensation of chromatin in CDA type I erythroblasts resembles the initial changes observed in cells undergoing apoptosis (Fig 1D). However, we failed to demonstrate DNA ladders in CDA type I BM. It was previously suggested that morphological features of apoptosis may occur in the absence of interchromosomal DNA fragmentation, which could be a later event in the apoptotic process.

Recently, S-phase arrest and apoptosis were described in overexpression of p34cdc2 at an inappropriate phase in the cell cycle. p34cdc2 is a serine threonine kinase, one of the newly reported cyclin-dependent kinases (cdks) that drive the cell cycle. When p34cdc2 complexes with cyclins A and B, it controls cell entry into mitosis. Premature activation of p34cdc2 promotes dissolution of the nuclear membrane and chromatin condensation. This may be a general mechanism by which cells undergoing apoptosis initiate disruption of the nucleus, as proposed by Shi et al. Further studies are indicated to better characterize the role of erythroid S phase arrest and apoptosis and their relation to the basic defect in CDA type I.

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