

DIAMOND-BLACKFAN ANEMIA: A CONGENITAL DEFECT IN ERYTHROPOIESIS

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ABSTRACT

Diamond-Blackfan anemia (DBA) is a congenital pure red blood cell aplasia diagnosed in the first year of life. Familiarity is apparent in 10% of patients, with all other cases being sporadic. Physical abnormalities are present in at least one third of patients, pointing to a defect in early embryo development. The main clinical sign is profound isolated anemia, with normal numbers and functioning of the other hemopoietic cells. Reticulocyte counts are very low. Bone marrow reflects defective erythropoiesis, showing a very low number of erythropoietic precursors and a reduction of BFU-E progenitor cells. Proliferation and differentiation of the other lineages are normal. The very high erythropoietin (EPO) levels are usually not proportionate to the level of anemia and reflect relative EPO insensitivity, which is also apparent *in vitro*. Conversely, erythroid progenitors from DBA patients also show a defective or incomplete response to other erythropoietic growth factors, such as IL-3 or IL-6. A significant response has been observed *in vitro* to stem cell factor in many, but not all patients.

Many patients respond clinically to corticosteroids and some develop hematologic remissions, both after corticosteroids and spontaneously. Patients who do not respond to corticosteroids and those who have to discontinue treatment because of side effects must rely on chronic transfusion and are thus exposed to all its complications. Bone marrow transplantation has been performed in some individuals, usually with a successful outcome. This suggests a normal marrow microenvironment and rules out the hypothesis of defective stromal cell function.

The variable clinical and biological patterns may be the expression of multiple etiologies or represent variable expressivity of a single genetic defect. Only identification of the responsible gene(s) will solve this question. Growth factors exerting an effect on erythropoiesis (and relative receptors) or transacting proteins which regulate their expression are likely candidates in the hunt for a causal gene.

Key words: Diamond-Blackfan anemia, erythropoiesis, growth factors

In 1938, Diamond and Blackfan reported four cases of red cell aplasia in early infancy.¹ They regarded this as a mild form of complete aplastic anemia and called it *hypoplastic anemia*. Two similar cases had been described in 1936 by Josephs.² The subsequent observation of many more patients resulted in a wide variety of names: congenital hypoplastic anemia, chronic

aregenerative anemia, erythrocytopenia, chronic idiopathic erythroblastopenia with aplastic anemia, Josephs-Diamond-Blackfan anemia, and Diamond-Blackfan anemia. Erythrocytopenia is probably the best description, but the disease is most commonly known as Diamond-Blackfan anemia (DBA).^{3,4} DBA and Fanconi anemia (FA)³ are the most

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common of many inherited bone marrow failure syndromes. Their many similarities once caused them to be considered variants of a single disorder. More recent biological and molecular studies have clearly differentiated them, as will be discussed later in this paper.

But is DBA really a single disease or a miscellany of different conditions sharing a common phenotype? Is it inherited or acquired, or both? Recent developments in the understanding and diagnosis of red cell aplasias, both acquired, such as those due to parvovirus B19 infection, and inherited, such as FA, have substantially improved the definition of DBA. This paper reviews the present position and possible future developments with regard to the etiology, pathogenesis, and treatment of this intriguing condition.

Inheritance

More than 400 cases of DBA have been reported (reviewed in ref. #3). Most patients are Caucasians, but Blacks, Indians and Japanese are also affected. Difficulties in diagnosis and the apparent rarity of DBA have precluded definition of its epidemiology. A retrospective study of children aged < 15 years in the northern health region of England over a 7-year period indicated an annual incidence of 1.5:1,000,000 live births,⁵ but more recent data presented at the annual meeting of the DBA working group of the *European Society for Pediatric Hematology and Immunology* indicate an incidence of 4-5 per million. Preliminary data from a census of congenital aplasias organized by our group in 1995 among the centers of the AIEOP (*Italian Association for Pediatric Hematology and Oncology*) reported a prevalence of 1:300,000 in Italy.

Inheritance is not clear. Only 10% of patients show a family history for DBA.³ One third of these display evidence of dominant inheritance; the disease is present in one parent (up to three consecutive generations in a single family have been observed) or in stepchildren, who are likely to have inherited the abnormal gene from their shared parent. The remainder show features of recessive inheritance such as consanguinity

among parents or more than one affected offspring in the family.

In the dominant families, DBA is usually more severe in the offspring than in the parents. The affected parent may have suffered anemia requiring transfusion and/or steroids during childhood, but subsequently achieved remission and was not under treatment when the disease was diagnosed in the offspring. The incidence of physical abnormalities seems equal in the inherited and the overall group.

The majority of patients are sporadic. DBA may thus be due to *de novo* mutations of a dominant gene, but this pattern could also fit recessive inheritance of a rare condition.

The male to female ratio is 1.1:1, thus excluding an X-linked condition. The reduced fitness further hampers the definition of inheritance. The penetrance is unknown. The expressivity seems widely variable, although multiple etiologies evolving into a similar phenotype cannot be ruled out.

Clinical findings

Hematological parameters

The main clinical symptom is anemia. This is often present at birth, and in any event appears in the first year of life in more than 90% of patients.^{3,4} The other hemopoietic lineages are by definition normal; slightly abnormal low leukocyte and high platelet counts have been reported, but they are neither constant nor persistent, and rarely relevant clinically. Conversely, thrombocytopenia and/or neutropenia may be encountered in the follow-up of transfused patients due to sensitization. The variable phenotypes have prompted very tight diagnostic criteria: 1) normochromic, usually macrocytic, occasionally normocytic, anemia developing in the first year of life; 2) reticulocytopenia; 3) normocellular bone marrow with a selective deficiency of red cell precursors; 4) normal or slightly decreased leukocyte counts; 5) normal or slightly increased platelet counts (Table 1).

Fetal Hb is usually increased and distributed heterogeneously, as expected in any type of bone marrow failure. Similarly, red cells from DBA

Table 1. Hematological features of DBA.

<ul style="list-style-type: none"> • macrocytic anemia developing in the first year of life • profound reticulocytopenia • normocellular bone marrow with selective erythroid deficiency • normal (or only slightly reduced) leukocyte count • normal (or only slightly increased) platelet count • increased erythrocyte ADA • increased HbF • increased EPO levels • high levels of serum B₁₂ and folate
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patients also show an increased *i* antigenicity, reflecting the reduced marrow transit time of the maturing cells.

Bone marrow shows normal cellularity, with normal myeloid and megakaryocyte precursors. Eosinophilia is sometimes present. The erythroid compartment is strikingly abnormal, ranging from erythroid hypoplasia to total aplasia in 90% of patients. Some patients show immature proerythroblasts as the only erythroid precursor cells. The remainder display either normal number and maturation of erythroblasts or erythroid hyperplasia with a maturation arrest and increased numbers of immature precursors.

Because of their defective erythropoiesis, DBA patients show increased values of folic acid, vitamin B₁₂ and especially erythropoietin (EPO), whose levels are higher than expected for the level of anemia. Ferritin and serum iron levels are usually high in heavily transfused patients, who are prone to all the complications of chronic transfusion.

In more than 90% of patients, red blood cell adenosine deaminase (ADA) ranges from 2 to 10 times higher than normal.⁶ A similar increase has also been found in a minority of the normal parents of children with DBA. Since this situation has also been found in children with acute lymphoblastic leukemia, it has been considered an indication of disordered erythropoiesis. Analysis of the electrophoretic pattern in two brothers with DBA who were compound heterozygotes for different alloenzymes showed that both alloenzymes were hyperactive.⁷ This behavior is suggestive of a transacting mech-

nism of activation, excluding a mutation at the ADA locus. On the other hand, abnormalities were observed in other red blood cell enzymes involved in purine or pyrimidine metabolism. In some cases, the elevation was consistent with the presence of young fetal-like erythrocytes.

Karyotype is usually normal. Chromosomes from DBA patients do not show increased breakage to diepoxbutane (DEB) and other alkylating agents. This feature differentiates DBA from FA. Sister chromatid exchange is normal.

A further heterogeneity is shown in their response to steroids and follow-up, as discussed in detail below. Life expectancy is better in patients responsive to corticosteroids, with predicted 50% survival being > 40 years as opposed to about 30 years in non-responders or untreated patients.³

Pregnancy has been reported in patients with a more favorable outcome. Their offspring are not usually affected. Pregnancy, however, is a further strain on the defective erythropoiesis, probably because bone marrow is inhibited by progesterone. In a few cases it has induced a relapse in women in apparent remission.

Progression to a hematological malignancy has been observed in at least eight patients: one developed acute lymphoblastic leukemia, three acute myeloblastic leukemia, a 14-month-old boy megakaryocytic leukemia, and three myelodysplastic syndromes, two of which progressed to myeloid leukemias.^{3,8,9}

Dysmorphic features

Physical abnormalities are present in at least one third of patients: in their review of 436 cases mainly from the literature, Alter and Young³ reported congenital anomalies in more than 100 patients (26%), whereas Janov *et al.*¹⁰ observed at least one anomaly in 34 of 76 patients (45%) diagnosed or followed at Children's Hospital, Boston, between 1931 and 1992. In the former series the percentage is most probably underestimated, since some of the reports in literature did not provide a physical description.

A distinct facial appearance was described by Cathie as «*tow colored hair, snub nose, thick upper lip, and an intelligent expression*». The Cathie facies has been observed in many patients who

resemble each other more than their own siblings. Often patients have more than one anomaly. Other head anomalies include micrognathia, microcephaly, dysmorphic features, cleft palate and/or lip, bifid uvula. Congenital heart disease and renal abnormalities are quite frequent. Eye anomalies include glaucoma, hypertelorism, cataract, microphthalmos, anophthalmos. The most common anomalies are defects of the upper limb, especially of the thumbs, including triphalangeal, bifid, accessory, absent, hypoplastic, subluxed thumbs, either unilaterally or bilaterally.^{3,10} Often the only physical abnormality is flattening of the thenar eminences or weakness of radial pulses, probably representing a milder expression of defective limb development. These signs may be missed by hematologists untrained in dysmorphology.

Differential diagnosis

The most common conditions presenting with isolated erythroid aplasia are listed in Table 2. The secondary and syndromic forms are usually easy to differentiate. Discrimination between DBA and transient erythroblastopenia of childhood (TEC) or parvovirus B19 infection may pose problems (Table 3). TEC usually appears after a viral infection in a child with previously normal erythropoiesis. It is thought

Table 2. Pure red blood cell aplasias.

<ul style="list-style-type: none"> • inherited pure red blood cell aplasia (Diamond-Blackfan anemia) • transient erythroblastopenia of childhood (TEC) • transient aplastic crisis of hemolysis (parvovirus B19 infection) • secondary to: <ul style="list-style-type: none"> hematologic malignancies solid tumors paraneoplastic syndromes autoimmune disorders viral infections pregnancy drugs • associated with syndromes

to be due to an autoimmune process affecting the early progenitor cells. Since it occurs frequently in infancy, differential diagnosis with DBA may be difficult, particularly when it is not possible to demonstrate normal erythropoiesis before onset. The absence of malformations and normal levels of adenosine deaminase (ADA) in TEC enable the two diseases to be distinguished, but *wait and see* is probably the best tactic. Some cases of TEC are due to parvovirus B19 infection via an autoimmune mechanism. This virus is the etiological agent of erythema infectiosum, a mild exanthema of childhood that

Table 3. Differential diagnosis between DBA, FA and acquired anemias.

	DBA	FA	acquired anemias
involved lineage	pure red cell aplasia	pancytopenia	pure red cell aplasia
viral agents	no	no	yes
age of onset	early infancy	late infancy	early infancy/fetal life
malformations	40%	80%	no
chromosomal breakage	no	yes	no
RBC ADA	high	normal	normal
response to corticosteroids	yes in 50%	poor	yes
response to immunosuppressive agents	no	no	yes
follow-up	remission/relapse/chronic	chronic	single episode
BFU-E	absent	absent	normal/slightly reduced
CFU-GM	normal	reduced	normal

progresses with influenza-like symptoms and a rash.¹¹ The virus has a tropism for erythroid precursor cells and binds to the P antigen, belonging to the P blood group. The proliferation of parvovirus B19 in these cells causes their destruction and is responsible for transient aplastic crisis of hemolysis. The few individuals who do not express the P antigen on their red blood cells (p phenotype) are naturally immune to parvovirus B19 infection.¹² Intrauterine infection with parvovirus B19 may result in hydrops fetalis or chronic pure red cell or tilineage aplasia.¹³ Serological as well as DNA based analyses identify the viral infection and should be performed before making a diagnosis of DBA.

DBA patients, like those with other diseases that affect erythropoiesis, show increased bone marrow sensitivity to parvovirus B19 infection. An apparent relapse in a DBA patient in remission due to this infection has been reported.¹⁴

It has also been proposed that intrauterine infection results in sporadic DBA. Migration of erythroid stem cells from the yolk sac to the liver and to prospective bone marrow sites occurs at the 5th week of gestation or shortly thereafter, and paddle-shaped limb buds become visible at the beginning of the 5th week.¹⁵ Red cell aplasia and hand abnormalities may be the outcome of an insult to the early embryo. However, either an infectious insult or genetic deprivation of an essential factor could be responsible for the same mechanism.

Genetic syndromes with disordered erythropoiesis include FA, Pears on's disease, and cartilage-hair hypoplasia.

The same dysmorphisms occur in FA and in DBA, but the number of anomalies and their severity are lower in DBA. Moreover, the frequency of anomalies associated with FA is much higher than in DBA (approximately 80%).³ The distinct FA hematological findings, i.e. pancytopenia, are usually decisive in diagnosis. However, in some cases FA may show a pure red cell aplasia at presentation with subsequent evolution to pancytopenia. The two diseases are eventually distinguished by the DEB assay, which is by definition normal in DBA. ADA values are normal in FA. Table 4 reports the most common dysmorphic features found in DBA

Table 4. Dysmorphic features in DBA and FA.

Abnormality	DBA	FA
abnormal pigmentation	–	>50%
head, face and palate	13%	39%
upper limbs	9%	50%
birth weight < 2500 g	8%	13%
short stature	6%	62%
eyes	6%	27%
renal	4%	24%
neck	4%	–
genitalia	3%	40% males, 3% females
retardation	3%	13%
skeletal	3%	16%
cardiopulmonary	2%	7%
other		3%
at least one anomaly	26%	80%

Data are from Young and Alter, who reviewed the literature data for 207 DBA and 838 FA patients (3). Several patients had more than one anomaly. The percentages are most probably underestimated since some reports did not provide a physical description.

and FA patients and their relative prevalences.

Pearson's syndrome is a fatal disorder due to mitochondrial DNA deletions. The disease is characterized by exocrine pancreatic dysfunction and severe macrocytic anemia at onset, but soon multiorgan involvement becomes apparent with variable combinations of ophthalmoplegia, mitochondrial myopathy, lactate elevation, neurologic, cardiac, endocrine, and/or renal manifestations (Kearnes-Sayre syndromes).¹⁶ Genetic transmission is matrilineal. ADA levels are usually normal. Myeloid and erythroid precursors show marked vacuolization. The disease does not respond to corticosteroids. The severe evolution and the presence of mitochondrial DNA mutations distinguish Pearson's syndrome from DBA.

Old reports describe DBA associated with achondroplasia.^{3,17} In most of these cases, however, the disease was not DBA but the rare cartilage-hair hypoplasia, which is an autosomal recessive metaphyseal chondrodysplasia with short-limbed short stature resembling achondroplasia, hypoplastic hair, defective immunity and erythropoiesis.¹⁸ This disease is more common among Finns and the Older Order Amish population. The gene responsible has recently been mapped on the short arm of chromosome 9.¹⁹

Biological studies

Physiology of normal erythropoiesis

In the bone marrow, pluripotent stem cells are capable of both self-renewal and differentiation into any one of the blood cell lines. When a stem cell begins differentiation it becomes directed toward the production of one or more cell lines, but it loses its pluripotent potential and the capacity for self-renewal. This progenitor cell has not yet acquired the specific features of the cell line to which it is committed and may be recognized only by its ability to give rise to a colony of differentiated progeny of that series in *in vitro* cultures. Several stages of differentiation are then needed to reach morphologically recognizable precursors of each cell line. The most mature progenitor-derived colonies are the first to develop into small colonies in culture, whereas the most immature progenitors take longer to mature and to develop into bigger colonies. Within this continuum, conventional classification of erythroid progenitor cells includes CFU-GEMM (granulocyte-erythrocyte-macrophage-megakaryocyte colony-forming units; or CFU-Mix), CFU-b/M/E (basophil-megakaryocyte-erythroid colony-forming units), BFU-E (burst-forming units-erythroid), and CFU-E (colony-forming units-erythroid). CFU-GEMM represent the common progenitor for the erythroid, myeloid, and megakaryocyte series. CFU-b/M/E is a common progenitor for the basophil, megakaryocyte and erythroid lineages. BFU-E, the most primitive pure erythroid progenitors, form very large colonies of thousands of nucleated erythroid precursors in culture, whereas CFU-E, the most differentiated erythroid progenitors, form small colonies of up to 64 nucleated precursors. Although lacking the capacity for prolonged self-renewal, BFU-E are capable of extensive proliferation, whereas CFU-E have a more limited capacity for amplification.²⁰

The morphologically recognizable erythroid precursors continue their process of maturation through subsequent stages, such as the nucleated pronormoblast and normoblast, to reach after extrusion of the nucleus the stage of reticulocyte. These cells have lost the capacity for self-renewal and CFU-E must continually differenti-

ate into pronormoblasts to replace those which have matured into later precursors. Each pronormoblast gives rise to 8 to 32 erythrocytes in 7 to 8 days.

The entire process is regulated by the successive participation of specific growth factors able to initiate and sustain the process to its end result, i.e. the formation of mature red blood cells. The factors able to initiate differentiation (once named burst promoting activity-BPA) are released by the marrow stromal cells and are regulated by local marrow conditions. Specific cellular interactions with membrane proteins or extracellular matrix proteins of stromal cells are probably involved in this process. Stem cell factor (SCF), IL-3, GM-CSF, IL-6 and IL-11 are among the early-acting growth factors.^{20,21} As differentiation progresses, sensitivity to these growth factors is progressively lost, whereas the more differentiated progenitors become increasingly responsive to the specific trophic hormone produced by the adult kidney: erythropoietin (EPO). This is obtained by the progressive loss with differentiation of cell surface receptors for early-acting growth factors and the acquisition of cell surface receptors for EPO. As a consequence, differentiation of BFU-E into CFU-E requires both early-acting factors and erythropoietin, while the most differentiated progenitors, CFU-E, are responsive only to EPO.

EPO also regulates the rate at which morphologically recognizable erythroid precursors mature and are released from the marrow.²⁰

Erythropoiesis in DBA

Bone marrow erythroid progenitors and recognizable precursors are reduced or absent in most DBA patients and show reduced growth in standard clonogenic assays.²²⁻²⁴ However, these assays give dissimilar results and their data cannot be regarded as pathognomonic or diagnostic. Colony numbers have no relationship to clinical presentation or to laboratory parameters such as hemoglobin (Hb) levels or percentage of marrow erythroid precursors.

Humoral or cellular suppression of hematopoiesis has been suggested, but has never been confirmed. A defect in the microenvironment seems to be ruled out by the good results

obtained with bone marrow transplantation.²⁵⁻²⁷ Thus the general consensus is that most cases are due to an intrinsic disorder of the erythroid progenitors, which do not respond normally to inducers of erythropoiesis. Accelerated programmed cell death (apoptosis) has been observed after EPO deprivation in erythroid marrow progenitors from DBA patients.²⁸ In many cell systems, apoptosis is induced when appropriate growth factors are withheld, and a role in protecting cells from apoptosis has been hypothesized for EPO.

In several laboratories, unusually high concentrations of EPO improved DBA colony cultures in some but not all cases.^{29,30} In general, a specific feature of DBA seems to be EPO insensitivity, as shown by *in vivo* EPO levels, which are not appropriate for the degree of anemia, and by the absent or limited response to EPO *in vitro*. Several investigators have demonstrated that burst-promoting activity (BPA) added to DBA bone marrow cultures increases erythroid growth and EPO sensitivity.^{30,31} The composition of BPA is unknown, but it is supposed to contain specific erythroid growth factors. Thus many investigators were prompted to evaluate the effect of different growth factors on DBA erythroid cultures. Halperin *et al.* reported that the size and number of marrow BFU-E was improved with IL-3 *in vitro*.³² However, the most striking results were observed by four independent groups when SCF was added to IL-3 and EPO. Bone marrow from 33 patients was treated and 30 responded, albeit to a different degree. Abkowitz *et al.* showed that four of four DBA bone marrow samples reached normal numbers of BFU-E with SCF.³³ Fourteen of 16 patients responded *in vitro* in the study by Alter *et al.*, and four of these normalized.³⁴ Olivieri *et al.* studied 10 patients and observed three types of response: normal numbers of BFU-E in four, moderate numbers in two, poor response in three, and one did not grow.³⁵ Three responders required high concentrations and three low concentrations of SCF. Bagnara *et al.* purified CD34⁺ progenitors from 10 DBA bone marrow samples and found reduced numbers of BFU-E in nine, similar to the results obtained with unpurified progenitors.³⁶ The three patients

treated with SCF achieved low normal numbers of BFU-E. Since these results were obtained from purified CD34⁺ cells, this study is the only one in which a coadjuvant effect from other marrow cell lines could be ruled out.

The heterogeneity in response to SCF seems related to age, being worse in older patients.³⁷ This suggested that the hemopoietic failure in DBA is a progressive defect with reduced growth factor response in progressively earlier cells from CFU-E to CFU-GEMM. This would also explain the occurrence of mild abnormalities in other lineages in the course of time.³⁸ On the other hand, it is possible that platelets and myeloid cells are affected by sensitization due to repeated transfusion.

Other growth factors affecting erythropoiesis have recently been identified, among them thrombopoietin (TPO)³⁹ and IL-9.⁴⁰ It will be interesting to observe whether these factors have an effect on DBA progenitor growth.

Low levels of flt3/flk2 ligand (FL) have been observed in serum from DBA patients as well as in other unilineal hemopoietic disorders such as thalassemia and TEC, whereas high levels have been found in FA and in acquired aplasia.⁴¹ Since FL is an early growth factor acting on granulocyte-megakaryocyte lineages, the low levels in DBA suggest the hypothesis of an etiologic agent acting selectively on the committed erythroid precursor.

Molecular findings and animal models

DBA patients usually display a normal karyotype. Non-specific chromosome abnormalities, including an achromatic area in chromosome 1, a pericentric inversion on chromosome 1 and an enlarged chromosome 16, have been described.³

Although some FA patients may have physical findings superficially resembling DBA, a search for chromosomal breakage enables the two diseases to be distinguished. Definition of the molecular basis of FA has made such great strides that molecular analyses might eventually be used to differentiate FA and DBA.⁴²⁻⁴⁴

The gene responsible for DBA, however, is still unknown. As mentioned earlier, candidate genes are those encoding for erythropoietic

cytokines and their receptors, or erythropoietic transacting genes, or even genes involved in the transduction of the message delivered by growth factors (Figure 1).

Many promising hypotheses have been disproved by clinical or biological information. Response to EPO is impaired in DBA both *in vivo* and *in vitro*, and serum EPO concentration is high in proportion to the severity of the anemia. The structure of EPO, however, is normal and anti-EPO antibodies are absent.⁴ EPO is therefore ruled out, whereas an abnormality in its receptor has frequently been suggested to explain the relative EPO insensitivity.⁴⁵ Several nonsense mutations in the EPO receptor gene (EPO-R) have been identified in familial erythrocytosis.^{46,47} All of them result in loss of the negative, but retention of the positive regulatory domain in the COOH terminal region, with the result that the mutated protein excessively stimulates erythropoiesis. Mutations in the positive regulatory domain of EPO-R, as well as those affecting a consensus sequence essential

for transduction of the receptor signal, such as those constructed by *in vitro* mutagenesis,⁴⁸⁻⁵⁰ could explain the phenotype of DBA.

We analyzed the coding sequence of the EPO-R gene for mutations in 23 DBA patients, but found only silent DNA changes. Moreover, using a highly polymorphic intragenic microsatellite, we observed an absence of concordant segregation of the EPO-R gene with the clinical DBA phenotype in two families. In one family, normal parents had three daughters with DBA and a single normal child, thus suggesting recessive inheritance and ruling out a *de novo* mutation. Mutations in the EPO-R were thus discarded as a common cause of DBA.⁵¹ On the other hand, abnormalities in EPO-R signal transduction or in an accessory protein cannot be excluded.⁵² Mice homozygous for a targeted null mutation within the EPO and the EPO-R genes, respectively, show reduced primitive erythropoiesis and die at day 13 to 15 of gestation due to complete failure of definitive erythropoiesis. Early (BFU-E) and late (CFU-E) com-

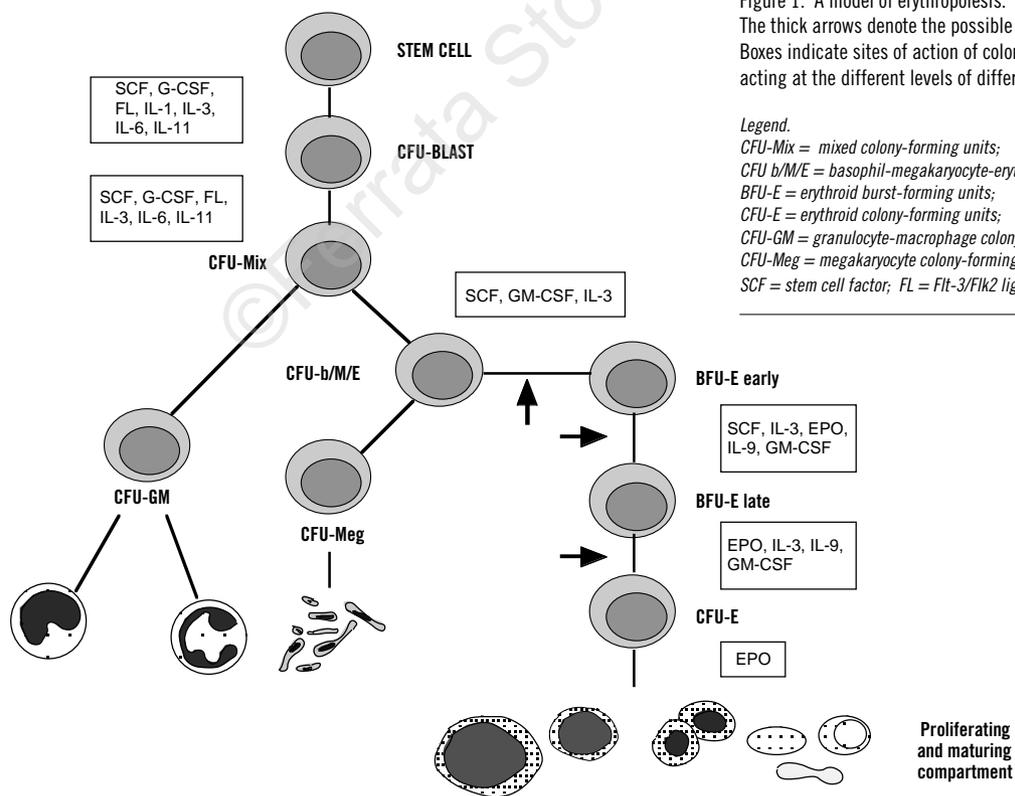


Figure 1. A model of erythropoiesis. The thick arrows denote the possible sites of blockage in DBA. Boxes indicate sites of action of colony-stimulating factors acting at the different levels of differentiation.

Legend.
 CFU-Mix = mixed colony-forming units;
 CFU b/M/E = basophil-megakaryocyte-erythroid colony-forming units
 BFU-E = erythroid burst-forming units;
 CFU-E = erythroid colony-forming units;
 CFU-GM = granulocyte-macrophage colony-forming units;
 CFU-Meg = megakaryocyte colony-forming units;
 SCF = stem cell factor; FL = Flt-3/Flk2 ligand

mitted progenitors were present in the fetal liver from both transgenic animals. This shows that neither EPO or EPO-R is required for the proliferation and differentiation of BFU-E to CFU-E progenitors. Conversely, EPO and EPO-R are crucial for the proliferation and survival of CFU-E progenitors and for their terminal differentiation into definitive erythrocytes.^{53,54}

Erythroid precursors from most DBA patients also do not respond to IL-3 or IL-6.³⁶ IL-3 has minimal effects when used in treatment, thus its gene is not a good candidate. In addition, its receptor and its transduction pathway are probably not involved, since DBA hematopoietic progenitors exposed to IL-3 are normally induced to differentiate towards the granulocyte and the megakaryocyte lineages.³⁶

In the early '90s, great enthusiasm was generated by the spectacular response shown by most DBA patients to SCF *in vitro*.³³⁻³⁶ Interestingly, a similar pattern was observed in Steel (Sl) mice, which have a clinical phenotype very similar to that of humans with DBA and carry deletions or point mutations within the Steel gene, the murine analog of the SCF gene.^{55,56} The intriguing hypothesis that the Steel mouse, which displays macrocytic anemia, lack of hair pigmentation, mast cell deficiency and sterility, might be a model for DBA prompted several laboratories to analyze the SCF gene extensively in DBA patients. However, the SCF transcript was found to be normal, both in quantity and in sequence, in several DBA patients, indicating that SCF itself is not directly involved.^{57,58} Mutations in the SCF receptor gene, *c-kit*, have been identified in another anemic strain of mice (the W mice), while in humans *c-kit* mutations have been identified in piebaldism, a disease with defective cutaneous and hair pigmentation but normal hematology.⁵⁹ These considerations militate against an involvement of the SCF/*c-kit* pathway in DBA. It is interesting to note that while both the W and Sl mice were relatively insensitive to EPO, they were also completely unresponsive to corticosteroids.⁶⁰ Thus neither of these genetically anemic strains of mice was a really good model for DBA.

Useful suggestions might arise from a study of knock-out mice lacking erythroid-specific tran-

scription factors. A growing body of evidence suggests that lineage commitment and differentiation are regulated by the combinatorial effects of multiple transcription factors on different target genes.⁶¹ Targeted mutations have been made in the murine genes that encode erythroid-specific transcriptional factors; the relative transgenic mice have provided further information on the functions of these proteins.

A pivotal role in controlling the expression of erythroid genes is played by the so-called GATA genes.⁶² GATA-1 is the founding member of family which includes a number of zinc-finger proteins that have been cloned on the basis of their sequence homology to GATA-1. GATA-1 specifically recognizes the GATA consensus sequence located in the promoters and enhancers of all the erythroid genes analyzed, including α - and β -globin genes, EPO and EPO-R. So far six GATA genes have been identified, all characterized by specific but overlapping expression. Of these only GATA-1, 2 and 3 are directly involved in hematopoiesis. Gene targeting in mouse embryonic stem (ES) cells at the GATA-1 locus has shown that GATA-1 is essential for erythroid cell development. No primitive erythroid progenitors are produced in the absence of GATA-1, but definitive precursors are produced at normal levels.⁶³ GATA-1⁻precursors are arrested at the proerythroblast stage and undergo premature cell death. No other cell type is affected. It is interesting to note that in chimeric embryos the presence of GATA-1⁺ erythroid cells is not able to compensate for GATA-1⁻ cells and the animals display a lethal anemia.

Disruption of GATA-2 results in the death of embryos at 10 days from severe anemia.⁶⁴ Primitive myeloid-erythroid precursors are produced in very reduced numbers, but T and B lymphocyte lineages also seem to be affected. Thus GATA-2 seems to control early events in the development of all blood cell lineages, affecting either the stem cell or early progenitors.

Transgenic mouse lacking GATA-3 have also been produced.⁶⁵ They too are severely anemic and die in utero at 11.75 days *post coitum*. Myeloid-erythroid progenitors are present in normal numbers in the yolk sac, but are very

much reduced in the fetal liver. GATA-3 is expressed in endothelial cells, T lymphocytes, placenta and the brain. Endothelial development seems to be affected mostly in these embryos, since lethality is probably due to massive internal bleeding. The role of GATA-3 in T-cell maturation could not be elucidated by the mutant animals because of their early death. Most interestingly, the mutated embryos show multiple morphological abnormalities, especially ones affecting the spinal chord, retina, and central nervous system. Thus GATA-3 is involved in controlling the differentiation of multiple tissue types.

The other GATA genes are mainly expressed by the developing heart and gut. It has been hypothesized that the loss of one of these genes might be partially compensated by upregulation of the others. Actually, GATA-1⁻ proerythroblasts transcribe higher than normal levels of GATA-2 and surprisingly normal levels of presumed GATA-1 target genes, including EPO-R. Forced erythroid expression of GATA-3 and GATA-4 in GATA-1-deficient ES cells rescues the mutant phenotype during their *in vitro* differentiation into blood cells.

Are knock-out mice for GATA genes good animal models for DBA? The most promising candidate should be the GATA-1 gene, but the human gene is located on the X chromosome. Thus its impairment in DBA would seem to be excluded on the basis of inheritance and the male/female ratio.

Chimeric mice lacking either GATA-2 or GATA-3 die very early in gestation, whereas the defect responsible for DBA does not appear to be so severe. The involvement of multiple tissues in GATA-3⁻ is a more suggestive phenomenon, even though the central nervous system is not generally impaired in DBA.

In conclusion, the study of transgenic mice might offer some good hints but is not expected to be conclusive, since there might always be extensive differences when trying to apply the information gathered to humans.

Analysis for mutations in candidate genes from DBA patients may offer some advantages with respect to classic whole genome linkage analysis. Mainly, the former may be performed

on DNA from all patients, either familiar or sporadic, since it takes into account possible genetic heterogeneity and the expected high number of *de novo* mutations. On the other hand, whole genome linkage analysis should be performed in selected families with definitely inherited DBA. Moreover, locus heterogeneity may hamper the recognition of linkage.

Another possible approach to elucidating the molecular basis of this disease might include the search for unstable DNA triplets in the dominant families.⁶⁶ A number of diseases characterized by unstable triplets have been identified in recent years, the most frequent being the fragile X syndrome.⁶⁷ These are usually autosomal dominant or X-linked and have the common feature of being progressively more severe in subsequent generations. This phenomenon, called *anticipation*, reflects the expansion of an intragenic triplet repeat (such as CAG or CGG) when transmitted from one generation to the next. The increased severity of the disease in the offspring of affected parents, observed within the DBA dominant families, as well as the finding of abnormal ADA levels in otherwise normal parents, might underscore a similar mechanism. The pooling of similar cases in an international collaborative study might allow a better definition of this phenomenon with a view to molecular studies.

Treatment

More than 50% of patients are responsive to steroids.³⁴ Some attain long periods of remission even after discontinuation of therapy. Spontaneous remissions have been observed not only at the beginning of steroid treatment, but also after months or years. However, eighty per cent of those who respond to corticosteroids become steroid-dependent and may experience steroid-related complications which necessitate discontinuation.

Treatment is usually started with 2 mg/kg/day prednisone for at least one month. If a hematological response is obtained, the dose may be gradually reduced either per day or every two days. Steroid-related complications include a reduced growth rate. Thus the treatment may be

stopped for 6 to 12 months every 2-4 years to allow a growth catch-up.⁶⁸ Treatment with expensive recombinant growth hormone (GH) is limited to patients with a demonstrated defect in GH secretion. Extremely high-dose intravenous methylprednisone therapy (100 mg/kg/day for 3 days followed by slow tapering of the dosage) has recently been attempted in a small number of patients who did not respond to the standard protocol.^{69,70} Six out of nine and three out of eight patients showed sustained remission without the need for maintenance prednisone therapy in the two studies. Even though the treatment was well tolerated, all patients in the second study showed an increase in body weight and oral moniliasis; 1 patient was hospitalized because of hyperglycemia and 2 developed central catheter infections. Because of the potential complications of such drastic doses, we feel that this approach should only be attempted in candidates for bone marrow transplantation, or in chronically transfused patients when compliance to chelation is very poor.

How glucocorticoids stimulate erythropoiesis is unknown. Since they are well-known transcription regulators, a role in the expression of growth factor receptors has been hypothesized. It seems that the effect is not mediated by immunosuppression since other immunosuppressive drugs such as cyclophosphamide, cyclosporine and anti-thymocyte globulin have provided no real benefit.⁷¹

Great interest has been generated recently following the *in vitro* observation of precursor differentiation and proliferation when cocktails of growth factors were added to the medium. Clinical trials have been attempted using EPO, GM-CSF and IL-3.⁷²⁻⁷⁷ Only IL-3 has shown some effect; the response ranged from 0/13 in the trial reported by Olivieri to 2/6 in the group studied by Dunbar. A significant reduction of transfusion requirements was observed in about 15% of the patients treated.⁷²⁻⁷⁷ Common side effects were fever, cutaneous reactions, headaches, chills, eosinophilia; some patients developed fluid retention. Remission was shown in sporadic patients after IL-3. Thus it has been suggested that IL-3 administration should be attempted in patients who do not respond to

steroids.

A compassionate trial with SCF is advocated by many hematologists involved in the treatment of patients with DBA, given the promising *in vitro* results. However, preliminary trials in cancer patients have occasionally shown severe allergic-like reactions and careful patient selection is essential.^{78,79}

In conclusion, chronic red cell transfusions in combination with iron chelating therapy or allogeneic bone marrow transplantation (BMT) from an HLA-identical sibling are the only treatment options for steroid-resistant patients.²⁵⁻²⁷ So far a small cohort of patients has received BMT and the results are similar to those observed in other congenital diseases, such as the thalassemias. Long-term disease-free survival has been shown in HLA-identical recipients. The similarity with thalassemia suggests a better outcome when transplants are performed before extensive transfusions are given. This is due to less sensitization to transplant antigens and reduced exposure to viruses and iron overload.²⁵ It has been suggested that BMT might be considered in patients who do not respond or become refractory to steroids, after attempting growth factor therapy, but before excessive transfusions have occurred. Unfortunately, the rarity of DBA precludes randomized trials and information on the outcome of BMT in DBA can only be collected by pooling international results.

In summary, the differences in genetics, clinical appearance, and biological *in vitro* studies suggest multiple etiologies, all affecting the erythroid precursor. The quality of life is good in patients who respond to steroid therapy and in the many individuals who experience remissions. On the other hand, both the expectancy and the quality of life are very much reduced in non-responders. Definition of the underlying molecular mechanism might completely change this scenario, especially if an administrable factor is found to be involved.

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