UvA-DARE (Digital Academic Repository)

Hematologic abnormalities in Shwachman Diamond syndrome: lack of genotype-phenotype relationship

Kuijpers, T.W.; Alders, M.; Tool, A.T.J.; Mellink, C.H.M.; Roos, D.; Hennekam, R.C.M.

DOI

[10.1182/blood-2004-11-4371](https://doi.org/10.1182/blood-2004-11-4371)

Publication date

2005

Published in

Blood

[Link to publication](#)

Citation for published version (APA):

Kuijpers, T. W., Alders, M., Tool, A. T. J., Mellink, C. H. M., Roos, D., & Hennekam, R. C. M. (2005). Hematologic abnormalities in Shwachman Diamond syndrome: lack of genotype-phenotype relationship. *Blood*, 106(1), 356-361. <https://doi.org/10.1182/blood-2004-11-4371>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Hematologic abnormalities in Shwachman Diamond syndrome: lack of genotype-phenotype relationship

Taco W. Kuijpers, Mariel Alders, Anton T. J. Tool, Clemens Mellink, Dirk Roos, and Raoul C. M. Hennekam

Shwachman-Diamond syndrome (SDS) is an autosomal-recessive disorder characterized by short stature, exocrine pancreatic insufficiency, and hematologic defects. The causative *SBDS* gene was sequenced in 20 of 23 unrelated patients with clinical SDS. Mutations in the *SBDS* gene were found in 75%, being identical in 11 patients. Hematologic parameters for all 3 lineages were determined over time such as absolute neutrophil counts (ANCs), granulocyte functions, and erythroid and myeloid colony formation (erythroid burst-forming unit [BFU-E] and

granulocyte-monocyte colony-forming unit [CFU-GM]) from hematopoietic progenitor cells, percentage of fetal hemoglobin (HbF), and platelet counts. Persistent neutropenia was present in 43% in the absence of apoptosis and unrelated to chemotaxis defects (in 65%) or infection rate. Irrespective of the ANC in vivo, abnormal CFU-GM was observed in all patients with SDS tested (14 of 14), whereas BFU-E was less often affected (9 of 14). Cytogenetic aberrations occurred in 5 of 19 patients in the absence of myelodysplasia. One child died during allogeneic bone

marrow transplantation. In conclusion, neutropenia and defective chemotaxis did not result in severe clinical infection in SDS. CFU-GMs were impaired in all patients tested. From the *SBDS* sequence data, we conclude that in patients with genetically proven SDS a genotype-phenotype relationship in SDS does not exist in clinical and hematologic terms. (Blood. 2005;106:356-361)

© 2005 by The American Society of Hematology

Introduction

Shwachman-Diamond syndrome (SDS; OMIM 260400)¹ is an autosomal, recessively-inherited disorder showing a wide variety of abnormalities and symptoms.² It is mainly characterized by short stature, exocrine pancreatic insufficiency, and bone marrow dysfunction.³⁻⁵ Several studies have shown that, with advancing age, 40% to 60% of patients become pancreatic sufficient. Elevated liver enzymes and hepatomegaly have been observed in the first years of life with subsequent improvement over the years.⁵

Intermittent neutropenia is the most common hematologic finding in SDS. Hematologic manifestations other than neutropenia include anemia, raised fetal hemoglobin (HbF) levels, thrombocytopenia, and, finally, aplastic anemia.^{4,6,7} Granulocyte colony-stimulating factor (G-CSF) has been used in some SDS subjects with severe neutropenia but is not generally advised because of the potential risk of acute myeloid leukemia.⁴

Growth retardation is the third typical manifestation: weight and length are already deficient at birth and remain decreased over time. A broad spectrum of skeletal abnormalities has been observed, of which metaphyseal dysplasia of ribs and long bones is the most common symptom. Several additional, less frequently observed clinical features such as heart failure or psychomotor retardation have been described.^{8,9}

Although the genetic basis of this rare disease has recently been described, no unifying pathogenic mechanism(s) has yet been shown to be responsible for SDS.¹⁰ Indirect lines of evidence from orthologs such as *YLR022c*, indicate that the *SBDS* gene may

encode an RNA-processing enzyme.¹¹ Seventy-five percent of the alleles in SDS were the result of a gene conversion confined to a short segment of about 240 base pairs (derived from an inactive pseudogene). Of the affected individuals, about 90% carry 1 and 60% carry 2 converted alleles. Alleles from affected individuals without conversion mutations had other changes in the coding region of *SBDS* that led to frameshift and missense changes.¹⁰

Neutrophils are produced in large numbers every day in the bone marrow, being predisposed to demise by apoptosis, a process that prevents the cytotoxic contents from the neutrophil granules to be released into the surrounding tissues and facilitates the harmless elimination of these cells by tissue macrophages.¹² Aging of normal neutrophils is accompanied by a progressive loss of functions, such as adherence, chemotaxis, and respiratory burst.^{13,14} We have studied the functional characteristics of neutrophils in other syndromes with concomitant neutropenia,^{15,16} and others have reported that in glycogen storage disease type 1-b (GSD1b) the functional defects of circulating neutrophils were largely due to premature apoptosis.¹⁴ In SDS, apoptosis has been noticed before in cultures of hematopoietic stem cells.¹⁷ However, apoptosis may be eminent at early stages only, whereas the mature circulating neutrophils are not affected and still functional.

To investigate the functional characteristics of neutrophils in SDS more closely, we sequenced the *SBDS* gene in a group of patients clinically diagnosed with SDS. We investigated long-term hematologic parameters, colony-forming potential of committed

From the Emma Children's Hospital, Academic Medical Center (AMC), University of Amsterdam, Amsterdam, The Netherlands; the Sanquin Research at CLB, Landsteiner Laboratory, University of Amsterdam, Amsterdam, The Netherlands; and the Department of Clinical Genetics, Institute for Human Genetics, AMC, Amsterdam, The Netherlands

Submitted November 16, 2004; accepted January 14, 2005. Prepublished online as *Blood* First Edition Paper, March 15, 2005; DOI 10.1182/blood-2004-11-4371.

Reprints: Taco W. Kuijpers, Emma Children's Hospital, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; e-mail t.w.kuijpers@amc.uva.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology

Table 1. Patient characteristics

Patient (sex)	Age, y	SBDS mutation	Clinical symptoms					Infection†	CFU-GM‡	Cytogenetics§	Hb, g/dL, and HbF (%)	Platelets, 10 ⁹ /L	Chemotaxis					
			Skin lesions	Hepatitis	Hepatomegaly	Diarrhea (elastase)*	Growth						Skeletal defects	ANC, µL	C5a	IL-8	PAF	
1 (F)	22	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	–	+	–	– (<5)	<p3	Coxa vara Femora	+	5	30	A	12.0 (2.1)	50-150	<600	N	N	N
2 (M)	2	NT	–	+	+	– (<5)	p10	–	–	–	–	–	11.4 (3.2)	300-450	>1200	N	N	N
3 (M)	3	NT	–	–	–	+ (5)	<<p3	Dig IV-V left hand	–	–	–	–	12.1 (1.0)	300-450	>1200	47	–	21
4 (M)	10	297-300 del AAGA [E99fs] IVS2 (258)+2T>C [C84fs]	+	–	–	±(15)	p25	Femora	–	20	75	N	13.1 (6.8)	50-300	<600	N	N	N
5 (F)	2	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	+	+	±	±(12)	p50	–	+	58	15	N	8.6 (16.9)	50-300	<600	N	N	N
6 (F)	35	NT	–	–	–	– (25)	p10	–	–	–	–	N	12.0 (0)	150-300	600-1200	34	52	40
7 (F)	<1	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	+	+	+	+ (<5)	<<p3	Multiple	+	86	30	N	9.0 (2.0)	50-150	<600#	10	12	20
8 (M)	10	IVS2 (258)+2T>C [C84fs]	–	+	±	+ (<5)	p3	Pectus excavatum	–	35	25	N	12.8 (1.2)	50-150	<600-1200	60	N	69
9 (M)	7	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	±	+	+	+ (<5)	p25	Pectus excavatum Femora	+	–	–	–	11.5 (1.0)	50-300	600-1200	40	45	44
10 (M)	20	No mutation	–	+	–	– (15)	p10	–	–	110	56	N	12.0 (0)	50-150	600-1200	N	63	66
11 (M)		No mutation	–	+	+	+ (14)	p50	Femora	–	216	14	N	11.7 (3.0)	50-150	>1200	32	22	N
12 (F)	5	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	–	–	+	– (30)	p10	–	–	52	23	N	12.8 (1.5)	150-300	>1200	N	N	N
13 (M)	18	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	–	+	–	– (15)	<p3	–	–	214	83	N	12.7 (1.0)	150-300	<600	29	53	58
14 (M)	4	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	–	+	+	+ (15)	p3	Multiple	+	43	10	A	7.5 (6.7)	300-450	<600-1200	66	38	41
15 (F)	18	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	+	–	–	±(30)	<p3	Syndactyly	±	44	3	A	12.0 (1.0)	150-300	<600-1200	N	N	–
16 (M)	16	183-184 TA>CT [K62X] IVS2 (258)+2T>C [C84fs]	–	–	–	± (<5)	<<p3	Scoliosis Femora	–	22	5	N	12.0 (0.1)	50-150	<600	N	32	64
17 (F)	4	297-300 del AAGA [E99fs] IVS2 (258)+2T>C [C84fs]	–	+	+	+ (<5)	p10	–	–	–	–	N	12.8 (0)	50-150	600-1200	58	60	65

(continued)

Table 1. Patient characteristics

18 (F)	4	183-184 TA>CT	-	+	-	+	(-5)	p<3	-	N	11.9 (0)	150-300	<600					
		[K62X] IVS2																
19 (M)	<1	No mutation	-	+	+	+	+(15)	p3	Femora	N	11.2 (0)	150-300	600-1200	30	49	44		
20 (F)	25	250 T>C [C84R]	+	+	+	+	+	p10	Femora		10.4 (3.1)	50-150	<600					
		IVS2 (258)+2T>C																
		[C84fs]																
21 (M)	16	No mutation	-	+	-	-	-(60)	p10	Multiple	189	12.4 (1.0)	150-300	<600	N	N	N		
22 (M)	<1	183-184 TA>CT	+	+	-	-	+	<p3	Femora	132	11.7 (0)	50-300	<600	A	30			
		[K62X] IVS2																
		(258)+2T>C[C84fs]																
23 (M)	7	No mutation	+	-	-	-	-(5)	p3	Pectus excavatum Dig II-IV left hand	A	12.7 (2.0)	50-300	>1200					

M indicates male; F, female.
*Elastase is expressed as µg/g feces (N < 200 µg/g).
†Infections are defined as follows: -, not present; ±, only at early age; and +, invasive or recurrent that required antibiotics.
‡Normal BFU-E values are 295 ± 100.8 colonies/10⁵ nucleated BM cells, CFU-GM values are 492 ± 120.2 colonies/10⁵ nucleated BM cells, age-matched.
§A indicates abnormal findings (see "Results and discussion"); N, normal findings.
||ANC indicates absolute neutrophil counts (low < 600 cells/µL, normal > 1200/µL).
¶Chemotaxis is expressed as percentage of the mean maximal slope (cells/min) of 2 age-matched controls measured on the same day (<70% of the mean of the day as well as <70% of the mean of 40 historic controls is considered abnormal; blanks without stimuli were not subtracted (see Figure 1A).
#G-CSF 30 µg/kg was given every other day.

hematopoietic progenitor cells, cytogenetic abnormalities of bone marrow (BM) cells, and a variety of characteristic granulocyte functions, as well as signs of early neutrophil apoptosis, to establish a genotype-phenotype relationship in these patients.

Patients, materials, and methods

SDS patient definition and inclusion

Patients were defined by the following major criteria: (1) early gastrointestinal symptoms (fat malabsorption and/or unexplained decrease in serum levels of fat-soluble vitamins, low fecal levels of elastase and/or chymotrypsin in the presence of normal sweat tests to exclude cystic fibrosis), (2) hematologic findings (unexplained anemia, low neutrophil and/or platelet number), (3) radiologic findings compatible with Shwachman-Diamond syndrome,¹⁸ and (4) growth failure. For a diagnosis of SDS, evidence of pancreatic exocrine dysfunction is obligatory at an early age. The presence of 2 major criteria made the diagnosis probable, and of 3 criteria definite, irrespective of the genetic analysis of the *SBDS* gene. Minor features were scored but not considered for the diagnosis. These features consisted of an unexplained hepatitis and/or hepatomegaly, skin manifestations, psychomotor skills, and behavioral difficulties. The study was approved by the institutional medical ethical committee of the AMC in accordance with the standards laid down in the 1964 declaration of Helsinki.

Molecular studies

Genomic DNA from peripheral mononuclear cells and fibroblasts from the patients were extracted by standard methods. All coding exons of the *SBDS* gene were amplified in separate polymerase chain reactions (PCRs). Sequencing was performed with the phycocerythrin (PE) dye terminator kit, and products were analyzed on an automated sequencer (ABI3100; Applied Biosystems, Foster City, CA). The genomic DNA was tested after written consent was obtained from the parent(s) and the patient (when older than 12 years of age). There were 3 refusals.

Hematologic studies

Morphologic analysis demonstrated hypocellular BM in smears, in some patients confirmed by BM biopsies. No excess of collagen or signs of fibrosis, disturbed bone marrow stroma development, or disorganized hematopoiesis was observed. Absolute numbers of progenitor B cells (CD19⁺, CD10⁺, CD24⁺), T cells (CD2⁺, CD3⁺, CD4⁺, CD8⁺), natural killer (NK) cells (CD3⁻, CD16⁺, CD56⁺), and myeloid cells (CD15⁺, CD14⁺, CD16⁺, CD65⁺) were determined by standard fluorescence-activated cell scan (FACScan) procedures. Colony-forming units of the erythroid and granulocyte-macrophage progenitors (erythroid burst-forming unit [BFU-E] and granulocyte-monocyte colony-forming unit [CFU-GM]) were determined in a 10- to 14-day semisolid culture and compared with normal age-matched values.

Cytogenetic analysis

Freshly obtained bone marrow samples were collected, cultured (without stimulation) in RPMI-1640 supplemented with 15% fetal calf serum for 24 hours, and harvested according to standard protocols. Phytohemagglutinin (PHA)—stimulated peripheral blood lymphocytes were cultured for 72 hours. Metaphase chromosomes were analyzed with a routine Q-banding method (QFA), and abnormal clones were defined according to the International System for Human Cytogenetic Nomenclature.¹⁹

Neutrophil purification and functional testing

Heparinized venous blood was collected from healthy donors and from patients with SDS, after obtaining informed consent. Granulocytes were isolated as described.^{15,16} Purity was always greater than 95%. In some experiments whole leukocyte preparations were used from which the erythrocytes were lysed by ice-cold isotonic NH₄Cl solution.^{15,16}

Neutrophil migration was assessed by means of the Fluoroblok inserts (Falcon; Becton Dickinson, San Jose, CA). Cells ($5 \times 10^6/\text{mL}$) were labeled with calcein-AM (1 μM final concentration; Molecular Probes, Leiden, Netherlands) for 30 minutes at 37°C , washed twice, and resuspended in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer at a concentration of $2 \times 10^6/\text{mL}$. Chemoattractant solution (platelet activating factor [PAF], interleukin 8 [IL-8], and complement 5a [C5a], all at 10 nM) or medium alone (0.8 mL/well) were placed in a 24-well plate, and 0.3 mL cell suspension was delivered to the inserts (3- μm pore size) and placed in the 24-well plate. Cell migration was assessed by measuring fluorescence in the lower compartment at 2.5-minute intervals for 45 minutes with the HTS7000+ plate reader (Perkin Elmer, Norwalk, CT). Maximal slope of migration was estimated over a 10-minute interval.

Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activity was assessed as hydrogen peroxide production determined by an Amplex Red kit (Molecular Probes). Neutrophils ($1 \times 10^6/\text{mL}$) were stimulated with 1 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), 10 ng/mL serum-treated zymosan (STZ), or 100 ng/mL phorbol 12-myristate 13-acetate (PMA), in the presence of Amplex Red (0.5 μM) and horseradish peroxidase (1 U/mL). Fluorescence was measured at 30-second intervals for 20 minutes with the HTS7000+ plate reader. Maximal slope of H_2O_2 release was assessed over a 2-minute interval.

Annexin-V binding, mitochondrial staining, and morphology were performed exactly as described.^{20,21} Morphology was determined after Giemsa staining of cytospin preparations. Apoptotic morphology was defined as the presence of condensed nuclei and simultaneous loss of the polysegmented nuclear appearance.

Statistics

Statistical analysis was performed with the SPSS package for Windows, version 10.0 (SPSS, Chicago, IL). For normally distributed data the Student *t* test was used to compare group means, otherwise the Mann-Whitney *U* test was applied. A 2-sided *P* value of less than .05 was considered statistically significant.

Results and discussion

The *SBDS* gene was sequenced in all but 3 cases clinically diagnosed as SDS. These latter 3 patients had gastrointestinal features as well as hematologic findings compatible with SDS (Table 1). Other causes had been excluded. In the other 20 patients, genetic abnormalities were found in the majority of patients. There were only a limited number of mutations present in our cohort. One mutation had not been reported until recently in Japanese patients with SDS as the 292-295 del AAAG,²² annotated here as the 297-300 del AAGA [E99fs] mutation in exon 3, resulting in a frameshift and premature stop codon. The other is a unique point mutation at codon 250T>C [C84R], that is predicted to result in a single amino acid substitution at a highly conserved position. This change was absent in DNA from more than 100 healthy control subjects, excluding the possibility of a common polymorphism. Normal *SBDS* genes were found in 5 patients (25%), comparable with other patient cohorts described thus far.^{10,22,23} In 1 patient we found only 1 mutation (patient 8).

Growth failure resulted in an extremely short stature in some patients, but was never disproportionate of nature. As indicated, radiologic investigations were performed in all patients, including chest, spine and hips, and upper and lower limbs including hands and feet. In 15 of the 23 patients (65%), defects were established (Table 1). The lack of abnormalities in some of the patients may be related to the age of radiologic scoring.¹⁸

Hepatitis, hepatomegaly, or both were commonly present, not necessarily combined. Hepatitis was limited to increased levels of

liver enzymes that were moderately increased (up to 300 U/mL) and in all patients gradually decreased over a variable period of months to years. When liver biopsies were performed during diagnostic procedures at the time SDS had not yet been considered, histologic findings of steatosis were observed without features of inflammation or fibrosis.

Elastase (and chymotrypsin) was tested in fecal samples. In all patients these were under the cut-off of 200 $\mu\text{g/g}$ feces, irrespective of the substitution of pancreatic enzymes or the patient's age at fecal sampling, even though some clinical resolution of the pancreatic insufficiency has been described in SDS.⁴ Gastrointestinal symptoms varied between patients and within the individual patient. For instance, the patient with congenital aplastic anemia (patient 7) initially had signs of diarrhea (passing of loose stools > 4 times a day) and developed eczematous skin lesions after birth suspect of hyperreactivity toward cow-milk protein. Symptoms did not disappear upon hydrolyzed formula feeding. After several months the eczema-like skin lesions resolved spontaneously, as more often observed in the patients with SDS mentioned here, and her stools became so compact that the girl was constipated for several weeks.

The number of red cells and thrombocytes did not fluctuate much apart from periods of mild infections (patients 4, 5, 7, 14, 15). Some of the patients had low platelet counts for a long time without major bleedings except for the patient with aplastic anemia.

Some had a mild anemia most often normocytic without abnormal levels of iron or iron-binding capacity of plasma, folic acid, or vitamin B-12 levels. Four patients had a macrocytic anemia and an increased HbF% on Hb electrophoresis (patients 2, 4, 5, and 14). As reported before, the patient with congenital anemia was on epopoietin but had a normocytic anemia without any increments in HbF compared with age control subjects.²⁴

Hematologic variation was observed in their blood counts (Table 1). As known from previous reports, the neutrophil counts may fluctuate in SDS.²⁻⁵ We classified the patients into 3 groups according to their ANC (serious neutropenia with < 600 polymorphonuclear neutrophils [PMNs]/ μL , moderate neutropenia with 600-1200 PMNs/ μL , and no relevant neutropenia with > 1200 PMNs/ μL). Neutrophil counts were normal in 5 patients and moderately affected in 8 patients. Some of the patients became seriously neutropenic during infections only. Neutropenia and circulating myeloid progenitor cells disappeared spontaneously at the time the infection resolved (patients 8, 14, 15). In 10 patients a severe chronic neutropenia for more than 6 months was present (patients 1, 4, 5, 7, 13, 16, 18, 20, 21, 22). One patient was put on G-CSF (patient 7; up to 30 $\mu\text{g/kg}$, 3 times a week) because of repeated infections resulting from congenital aplastic anemia.²⁴ The patient's response to G-CSF did not result in a significant rise in circulating neutrophil numbers, although her clinical condition improved considerably. Apart from this single case, we may conclude that the rate of infection was not strongly increased in our cohort.

We tested both neutrophil numbers and functions in 20 patients with SDS (Table 1). Directed cell motility (chemotaxis) of neutrophils can be disturbed in SDS.^{4,8,25,26} We tested chemotaxis toward a variety of neutrophil-specific stimuli, ie C5a, IL-8, or PAF. In 13 of 20 patients motility was diminished (65%) (Table 1). The activity toward different chemoattractants showed individual variation but as a patient group were defective toward all 3 stimuli (Figure 1A). In general, the variation in motility became more evident with the less potent stimulus. The rank order of potency in the chemotaxis assay used is as follows: C5a more than PAF more than IL-8. Also in control subjects, the IL-8-mediated chemotaxis is particularly prone to variation, due to minor illnesses such as common cold or unknown subclinical events. Because of this variation, we may explain that the individual patient not always differed

Figure 1. Myeloid defects in SDS. (A) Granulocyte chemotaxis toward rC5a. (B) NADPH-oxidase activity upon addition of serum-treated zymosan (STZ). Both tests compare purified neutrophils from patients with SDS and control subjects ($n = 40$). (C) BFU-E and CFU-GM of BM samples obtained from patients with SDS compared with healthy pediatric control subjects ($n = 20$) and children with benign autoimmune neutropenia of childhood (AINP; $n = 6$). The data are summarized from measurements performed over 5 years of study follow-up. Each patient is represented once. Some have been tested more often, in that case the mean of the experiments is taken. Results are the mean of standard triplicates, and error bars indicate standard deviation. Significance is marked as † $P < .05$, * $P < .001$, and ** $P < .0001$.

with all 3 stimuli used from the controls of that day (taking 70% as cut-off as in Table 1). In a dose-response study over a log₃ range in 3 of the patients with SDS with an abnormal chemotaxis response we found similar bell-shaped curves as well as the same optimal concentration for the induction of motility in the patients' neutrophils compared with control cells (data not shown). Chemotaxis defects did not correlate with ANC scores (Table 1; $P = .8$), which may indicate that there is no altered motility because of the presence of younger and more immature neutrophils in these patients.

The reason for the neutropenia was investigated by the determination of Annexin-V binding to freshly isolated neutrophils or whole blood samples from these patients with SDS. In contrast to our observations in GSD1b or Barth syndrome,^{15,16} no enhanced Annexin-V binding or any other feature of premature apoptosis was detected in the freshly purified SDS neutrophils, as tested by mitochondrial staining, Bax translocation, or morphology (data not shown).

NADPH-oxidase activity was preserved in SDS neutrophils of all patients, when tested with a variety of stimuli. Similar findings were made for phagocytosis and killing of *Staphylococcus aureus* or *Escherichia coli* (data not shown). Apart from the lack of Annexin-V binding to the circulating blood cells in SDS, a normal NADPH-oxidase activity upon activation by STZ (Figure 1B) or the phorbol ester PMA, and phagocytosis also excluded a death-prone cell type in this disease. We, therefore, believe that the myeloid defect or neutropenia in patients with SDS is unrelated to enhanced cell death or peripheral clearance of the circulating neutrophils.

Neutropenia may be the consequence of bone marrow failure. Overt failure at birth was observed in a single of our SDS patients only.²⁴ Although transfusion-dependent aplastic anemia has been reported at later age during childhood and adolescence,^{7,8} we did not observe any features of imminent bone marrow failure in any of the other patients with SDS. Colony formation tests (CFU-GM and BFU-E) were performed in 14 patients. BM cells uniformly showed a defective maturation and outgrowth of committed progenitor cells in the hematopoietic lineages tested (Figure 1C), irrespective of the blood cell and differential counts in the circulation (Table 1).

Abnormal hematopoietic progenitor as well as stromal cell function has been previously reported in SDS,^{17,27} which would indicate that the in vitro culture conditions used remain poor estimations of in vivo BM functionality. However, the results demonstrate intrinsic growth defects, whereby CFU-GM from pediatric control subjects or children with benign autoimmune neutropenia of childhood show normal or slightly increased outgrowth (Figure 1C).

Although some of the BM smears showed hypoplasia, dysplastic features were not present. We observed abnormal cytogenetic findings in 5 of 19 patients with SDS (Table 1). In one case a characteristic isochromosome 7, i(7)(q10), was noted (patient 1). This abnormality seems to be unrelated to myelodysplasia or malignancy, as discussed by us and others before.^{28,29} Another patient is closely followed because of increased HbF% as a possible sign of marrow failure or dysplasia. BM smears and biopsies repeatedly excluded myelodysplasia. BM cells were tested 46, XY [22] / 45 (–7)[3] (patient 14). During the last year, the blood cell count and differentials have normalized (apart from persisting neutropenia) and HbF% decreased spontaneously. Variability in cytogenetic abnormalities is an intriguing aspect in SDS. Smith et al³⁰ reported the transient appearance of a 20q-deletion in a SDS patient with i(7)(q10) as a nonrandom secondary change in SDS. As described here, we observed del(20q) as the sole feature in the BM of 2 patients in the absence of i(7)(q10) (patients 15 and 20). In the adolescent patient the cytogenetic anomaly disappeared in 2 years. Aplasia, malignant transformation, or progressive disease remained absent during the recent 5 years of follow-up in this patient. Another patient had a mosaic 46, XY / 46, XY t(7;10) abnormality in his blood cells (patient 23); its relevance warrants further study for the potential involvement of the regulation of the *SBDS* gene or neighboring sequences.

In conclusion, a lack of concordance in hematologic findings among affected siblings and the large variability within a family was noticed before.⁵ At that time the gene responsible for SDS was not yet identified. Using sequence data from 20 of 23 patients with SDS, we may infer that SDS-like syndromes exist without mutations in the *SBDS* coding sequence as was suggested previously.^{10,22,23} In the patients with genetically proven SDS only a small series of mutations make up the genetic defect. Even though our conclusions are based on a relatively small number of patients, we may infer from the genetic homogeneity in our study that a clear genotype-phenotype relationship in SDS does not exist in terms of clinical and hematologic parameters.

Acknowledgments

We thank Drs AJ Verhoeven and J Taminiu for critically reading the manuscript. We thank Drs J van der Vlist, CMR Weemaes, M Peters, and HJ Verkade for their kind cooperation and sharing of patient data.

References

- National Center for Biotechnology Information. OMIM: online Mendelian inheritance in man. <http://www.ncbi.nlm.nih.gov/omim>.
- Shwachman H, Diamond LK, Oski FA, Khaw KT. The syndrome of pancreatic insufficiency and bone marrow dysfunction. *J Pediatr*. 1964;65:645-663.
- Smith OP. Shwachman-Diamond syndrome. *Semin Hematol*. 2002;39:95-102.
- Dror Y, Freedman MH. Shwachman-Diamond syndrome [review]. *Br J Haematol*. 2002;118:701-713.
- Ginzberg H, Shin J, Ellis L, et al. Shwachman syndrome: phenotypic manifestations of sibling sets and isolated cases in a large patient cohort are similar. *J Pediatr*. 1999;135:81-88.
- Barrios NJ, Kirkpatrick DV. Successful cyclosporin A treatment of aplastic anaemia in Shwachman-Diamond syndrome. *Br J Haematol*. 1990;74:540-541.
- Woods WG, Krivit W, Lubin BH, Ramsay NK. Aplastic anemia associated with the Shwachman syndrome. In vivo and in vitro observations. *Am J Pediatr Hematol Oncol*. 1981;3:347-351.
- Aggett PJ, Cavanagh NPC, Matthew DJ, Pincott JR, Sutcliffe J, Harries JT. Shwachman's syndrome. A review of 21 cases. *Arch Dis Child*. 1980;55:331-347.
- Mack DR, Forstner GG, Wilchanski M, Freedman MH, Durie PR. Shwachman syndrome: exocrine pancreatic dysfunction and variable phenotypic expression. *Gastroenterology*. 1996;111:1593-1599.
- Boocock GR, Morrison JA, Popovic M, et al. Mutations in SBDS are associated with Shwachman-Diamond syndrome. *Nat Genet*. 2003;33:97-101.
- Koonin EV, Wolf YI, Aravind L. Prediction of the archaeal exosome and its connections with the proteasome and the translation and transcription machineries by a comparative-genomic approach. *Genome Res*. 2001;11:240-252.
- Kuijpers TW. Clinical symptoms and neutropenia: the balance of neutrophil development, functional activity, and cell death. *Eur J Pediatr*. 2002;161: S75-S82.
- Whyte MK, Meagher LC, MacDermott J, Haslett C. Impairment of function in aging neutrophils is associated with apoptosis. *J Immunol*. 1993;150: 5124-5134.
- Dransfield I, Stocks SC, Haslett C. Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. *Blood*. 1995;85:3264-3273.
- Kuijpers TW, Maiani NA, Tool ATJ, et al. Apoptotic neutrophils in the circulation of patients with Glycogen Storage Disease type 1b (GSD1b). *Blood*. 2003;101:5021-5024.
- Kuijpers TW, Maiani NA, Tool ATJ, et al. Neutrophils in Barth Syndrome (BTHS) avidly bind Annexin-V in the absence of apoptosis. *Blood*. 2004;103:3915-3923.
- Dror Y, Freedman MH. Shwachman-Diamond syndrome marrow cells show abnormally increased apoptosis mediated through the Fas pathway. *Blood*. 2001;97:3011-3016.
- Mäkitie O, Ellis L, Durie PR, et al. Skeletal phenotype in patients with Shwachman-Diamond syndrome and mutations in SBDS. *Clin Genet*. 2004; 65:101-112.
- ISCN, Mitelman F, ed. An International System for Human Cytogenetic Nomenclature. Basel, Switzerland: S Karger AG; 1995.
- Maiani NA, Mul FPJ, van Buul JD, Roos D, Kuijpers TW. Granulocyte colony-stimulating factor (G-CSF) inhibits the mitochondria-dependent activation of Caspase-3. *Blood*. 2002;99:672-679.
- Maiani NA, Roos D, Kuijpers TW. Tumor necrosis factor α induces a caspase-independent death pathway in human neutrophils. *Blood*. 2003;101:1987-1995.
- Nakashima E, Mabuchi A, Makita Y, et al. Novel SBDS mutations caused by gene conversion in Japanese patients with Shwachman-Diamond syndrome. *Hum Genet*. 2004;114:345-348.
- Woloszynek JR, Rothbaum RJ, Rawls AS, et al. Mutations of the SBDS gene are present in most patients with Shwachman-Diamond syndrome. *Blood*. Prepublished on July 29, 2004, as DOI 10.1182. (Now available as *Blood*. 2004;104: 3588-3590).
- Kuijpers TW, Nannenberg E, Alders M, Bredius R, Hennekam RCM. Congenital aplastic anemia caused by mutations in the SBDS gene. *Pediatrics*. 2004;114:e387-391.
- Ruuter P, Savilahti E, Repo H, Kosunen TU. Constant defect in neutrophil locomotion but with age decreasing susceptibility to infection in Shwachman syndrome. *Clin Exp Immunol*. 1984; 57:249-255.
- Stepanovic V, Wessels D, Goldman FD, Geiger J, Soll DR. The chemotaxis defect of Shwachman-Diamond syndrome leukocytes. *Cell Motil Cytoskeleton*. 2004;57:158-174.
- Dror Y, Freedman MH. Shwachman-Diamond syndrome: an inherited preleukemic bone marrow failure disorder with aberrant hematopoietic progenitors and faulty marrow microenvironment. *Blood*. 1999;94:3048-3054.
- Maserati E, Minelli A, Olivieri C, et al. Isochromosome (7)(q10) in Shwachman syndrome without MDS/AML and role of chromosome 7 anomalies in myeloproliferative disorders. *Cancer Genet Cytogenet*. 2000;121:167-171.
- Mellink CH, Alders M, van der Lelie H, Hennekam RCM, Kuijpers TW. SBDS mutations and isochromosome 7q in a patient with Shwachman-Diamond syndrome, no predisposition to malignant transformation. *Cancer Genet Cytogenet*. 2004; 154:144-149.
- Smith A, Shaw PJ, Webster B, et al. Intermittent 20q- and consistent i(7q) in a patient with in Shwachman-Diamond syndrome. *Pediatr Hematol Oncol*. 2002;19:525-528.