

Severe glucose-6-phosphate dehydrogenase deficiency leads to susceptibility to infection and absent NETosis

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Background: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymatic disorder of red blood cells in human subjects, causing hemolytic anemia linked to impaired nicotinamide adenine dinucleotide phosphate (NADPH) production and imbalanced redox homeostasis in erythrocytes. Because G6PD is expressed by a variety of hematologic and nonhematologic cells, a broader clinical phenotype could be postulated in G6PD-deficient patients. We describe 3 brothers with severe G6PD deficiency and susceptibility to bacterial infection.

Objective: We sought to study the molecular pathophysiology leading to susceptibility to infection in 3 siblings with severe G6PD deficiency.

Methods: Blood samples of 3 patients with severe G6PD deficiency were analyzed for G6PD enzyme activity, cellular

oxidized nicotinamide adenine dinucleotide phosphate/NADPH levels, phagocytic reactive oxygen species production, neutrophil extracellular trap (NET) formation, and neutrophil elastase translocation.

Results: In these 3 brothers strongly reduced NADPH oxidase function was found in granulocytes, leading to impaired NET formation. Defective NET formation has thus far been only observed in patients with the NADPH oxidase deficiency chronic granulomatous disease, who require antibiotic and antimycotic prophylaxis to prevent life-threatening bacterial and fungal infections.

Conclusion: Because severe G6PD deficiency can be a phenocopy of chronic granulomatous disease with regard to the cellular and clinical phenotype, careful evaluation of neutrophil function seems mandatory in these patients to decide on appropriate anti-infective preventive measures. Determining the level of G6PD enzyme activity should be followed by analysis of reactive oxygen species production and NET formation to decide on required antibiotic and antimycotic prophylaxis. (J Allergy Clin Immunol 2016;■■■■:■■■■-■■■■.)

Key words: Glucose-6-phosphate dehydrogenase deficiency, chronic granulomatous disease, neutrophil extracellular trap formation, innate immunity, immunodeficiency, NADPH

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X-chromosomal glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymatic disorder of red blood cells in human subjects, affecting 400 million persons worldwide.¹ G6PD deficiency causes a spectrum of hemolytic syndromes, with hemolytic crises induced by infections, drugs, or chemicals. The World Health Organization has classified the different G6PD variants into 5 classes, according to the level of residual enzyme activity and severity of hemolysis. Severe defects are characterized by less than 10% of normal enzyme activity, leading to chronic hemolytic anemia (class I) or intermittent episodes of acute hemolysis (class II).

G6PD catalyzes the initial step of the pentose-phosphate pathway, oxidizing glucose-6-phosphate to 6-phospho-gluconolactone and reducing nicotinamide adenine dinucleotide phosphate (NADP⁺) to reduced nicotinamide adenine dinucleotide phosphate (NADPH). In erythrocytes the pentose-phosphate pathway confers protection against oxidant cell injury by means of NADPH-dependent provision of reduced glutathione. G6PD-deficient erythrocytes become depleted of glutathione, leading to oxidation of sulfhydryl-containing proteins, such as hemoglobin and membrane components, and culminating in erythrocyte rigidity and hemolysis.²

Abbreviations used

CGD:	Chronic granulomatous disease
DHR:	Dihydrorhodamine 123
G6P:	Glucose-6-phosphate
G6PD:	Glucose-6-phosphate dehydrogenase
MNC:	Mononuclear cell
NADP+:	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
NBT:	Nitroblue tetrazolium
NET:	Neutrophil extracellular trap
PMA:	Phorbol 12-myristate 13-acetate
ROS:	Reactive oxygen species

In leukocytes reduced G6PD activity can result in reduced NADPH oxidase activity as a consequence of reduced NADPH generation, particularly in patients with more severe enzyme deficiency, such as G6PD-Mediterranean.³⁻⁵ Impaired phagocyte NADPH oxidase activity results in defective production of reactive oxygen species (ROS), which is required for the microbicidal activity of phagocytes. Genetic defects of the NADPH oxidase cause the primary immunodeficiency chronic granulomatous disease (CGD), which is characterized by recurrent severe infections with bacteria and fungi⁶⁻⁸ that are linked to impaired formation of ROS-dependent neutrophil extracellular traps (NETs).

In contrast, G6PD-deficient phagocytes typically present normal bactericidal activity,^{4,5} and rarely, increased susceptibility to infection has been reported in G6PD-deficient patients with severe enzymatic deficiency.^{9,10} Here we describe 3 brothers with severe defects in G6PD associated with impaired NET formation and, consequently, increased susceptibility to infection associated with impaired NET formation. These children were first given a diagnosis of CGD based on a history of recurrent infections and reduced ROS production in 1 patient, pointing to the importance of considering G6PD deficiency as a differential diagnosis of CGD on the one hand and of antimicrobial prophylactic management of immunodeficient patients with severe G6PD deficiency on the other.

METHODS**Patient history**

Three brothers with G6PD deficiency from a nonconsanguineous white family were analyzed in this study after written informed consent was obtained from the parents and according to local ethical requirements (Ethikkommission Universität Ulm, #27/06). The mother originates from Macedonia and is a carrier of the disease, and the father originates from Germany.

P1, a boy (age, 10 years and 2 months) with Fallot tetrad and type III esophageal atresia, was given a diagnosis in the course of familial screening after G6PD deficiency diagnosis in his younger brother (P2) at age 9 years and 10 months. He had a history of recurrent tonsillitis and pneumonias (no microbes isolated) requiring oral or intravenous antibiotic treatment, with each episode associated with hemolytic crises. At age 8 years, he had EBV tonsillitis together with the concomitant presence of *Serratia marcescens* in nose and throat swabs. He had delayed wound healing and eosinophilic esophagitis.

Index patient P2 (age, 6 years and 5 months) was given a diagnosis of X-linked CGD based on the results of dihydrorhodamine 123 (DHR) testing at age 6 years and 4 months for a history of recurrent otitis media and 1 episode of *Serratia marcescens* abscessing lymphadenitis of the left axilla. After normal *CYBB* genetic analysis (encoding the NADPH oxidase subunit gp91phox),

X-linked CGD was excluded, and he was given a diagnosis of G6PD deficiency after G6PD enzyme activity measurements in erythrocytes. Mild hemolysis was noted during viral infections.

P3, a 10-month-old boy, was given a diagnosis of G6PD deficiency at age 9 months in the course of familial screening. He was asymptomatic apart from slight anemia (hemoglobin, 90 g/L).

P1 and P2 received antibiotic prophylaxis with co-trimoxazole for recurrent *Serratia* species infection without resulting hemolysis.

Genetic analyses revealed the presence of a Toledo-type G6PD mutation (496C>T resulting in Arg166>Cys substitution) in all 3 patients and the mother.¹¹

Quantification of NET formation

NET formation was quantified by measuring extracellular DNA release.⁷ Briefly, 50,000 neutrophils per well were stimulated with 40 nmol/L phorbol 12-myristate 13-acetate (PMA), 500 ng/mL LPS, 5 μ mol/L ionomycin, or 600 U/mL glucose oxidase (GO) (all from Sigma-Aldrich, Buchs, Switzerland) in a 96-well plate in the presence of 1 μ mol/L Cytox Green (Molecular Probes, Life Technologies, NY), and fluorescence (extinction, 485 nm; emission, 520 nm) was measured every 20 minutes for 3 hours at 37°C in a Mithras LB940 Fluorescence reader (Berthold Technologies, Regensburg, Switzerland). Cytox Green signal on cell lysis with 1 mg/mL saponin served as a control.

G6PD activity assay

G6PD activity was measured in erythrocytes, as previously described.¹² Protein extracts were prepared by means of ultrasound sonication of 5×10^6 granulocytes or mononuclear cells (MNCs) in PBS, followed by centrifugation and storage in aliquots at -80°C . Blood samples were diluted in 0.9 mol/L sodium chloride and centrifuged to form erythroid cell pellets to prepare protein extracts of erythrocytes. An aliquot of 30 μ L of this packed erythrocyte cell pellet was diluted in 300 μ L of water and vortexed for 30 seconds for erythrocyte lysis. Protein extract was centrifuged for 2 minutes at 11,000 rpm and stored in aliquots at -80°C . Protein contents were determined by using the Pierce BCA Protein Assay Kit (Fisher Scientific AG, Wohlen, Switzerland), according to the manufacturer's description. Granulocyte or MNC cell lysates containing 20 μ g of total protein or erythrocyte lysates containing 200 μ g of total protein were incubated with 41 mmol/L triethanolamine, 4.1 mmol/L EDTA, 0.5 mmol/L oxidized nicotinamide adenine dinucleotide phosphate (NADP+), 3.32 mmol/L maleinimide, and 0.62 mmol/L glucose-6-phosphate in a volume of 115 μ L at 37°C, and the enzymatic reaction was followed by measuring the absorbance at 340 nm over time.

Measurement of NADPH/NADP+ levels

NADPH and NADP+ levels were determined in protein extracts with an NADP/NADPH assay kit (Abcam, Cambridge, United Kingdom), according to the manufacturer's instructions, and results were normalized to the protein concentration determined by using the Pierce BCA Protein Assay Kit (Fisher Scientific AG, Wohlen, Switzerland).

Immunofluorescence and microscopy

For immunofluorescence staining, 4×10^5 neutrophils per well were seeded on polylysine (Sigma-Aldrich, St Louis, Mo)-treated 8-well chamber slides (Ibidi GmbH, Planegg/Martinsried, Germany) and stimulated with 100 nmol/L PMA for 10 minutes, 1 hour, or 3 hours. After treatment, cells were fixed in 3% paraformaldehyde (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 20 minutes at 4°C, permeabilized with 0.5% Triton-X100 (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 1 minute at room temperature, and then incubated with Image-iT FX signal enhancer (Invitrogen, Carlsbad, Calif), stained with anti-NE (Mouse Monoclonal anti-Neutrophil Elastase Antibody, clone 10D10, kindly provided by Arturo Zychlinsky¹³), washed, and stained with anti-mouse IgG Alexa Fluor 555

and 4',6-diamidino-2-phenylindole (both from Life Technologies Europe B.V., Zug, Switzerland). All washes were performed in PBS supplemented with 1% fish skin gelatin and 0.02% saponin. Slides were counterstained with 4',6-diamidino-2-phenylindole (Molecular Probes, Life Technologies) and mounted with 50% glycerol in PBS (Amimed/BioConcept, Allschwil, Switzerland). Cells were visualized through a $\times 63$ 1.4 NA oil immersion lens with an inverted CLSM Leica SP5 confocal microscope (Leica, Wetzlar, Germany).

Detection of ROS production

ROS production was analyzed by using a DHR oxidation assay, a nitroblue tetrazolium (NBT) reduction assay, and a cytochrome C assay, as previously described.^{14,15} Within the cytochrome C assay, the slope in ferro-cytochrome C kinetic (550 nm) allows for calculating the amount of ROS synthesized and expressed in micromoles of ROS per minute and 1E6 granulocytes.

Fluorescence-activated cell sorting analysis

Polyclonal anti-LC3B and Anti-Flavocytochrome b_{558} clone 7D5 were from MBL (Woburn, Mass). Fluorescence-activated cell sorting analyses were carried out, as previously described,¹⁴ in a Gallios cytofluorometer (Beckman Coulter GmbH, Krefeld, Germany) and analyzed with Kaluza software (Beckman Coulter GmbH).

Statistical analysis

NADP(H) concentrations were determined in independent duplicates and normalized to protein contents. Normalized NADP⁺ and NADPH concentrations of duplicates were expressed as averages. For each duplicate, the SDs of NADP⁺ and NADPH values were calculated. NADP⁺ and NADPH levels of independent patient duplicate samples were compared with levels in healthy control samples ($n = 8$) in a 2-tailed unpaired Student *t* test. NET formation was determined in triplicates and expressed as averages. For statistical analysis of NET formation, cumulative data of 24 healthy control subjects were compared with those of the 3 G6PD-deficient brothers and with 3 independent analyses of the carrier mother conducted on 3 different days by using 2-tailed unpaired Student *t* tests of the time points.

RESULTS

Absent G6PD activity in granulocytes and erythrocytes of the 3 patients

We tested protein extracts derived from granulocytes, blood MNCs, and erythrocytes for G6PD activity. Cellular protein extract was supplemented with glucose-6-phosphate (G6P) and NADP⁺. The NADP⁺-dependent conversion of glucuronate-6-phosphate to ribulose-5-phosphate by glucuronate-6-phosphate dehydrogenase activity was inhibited in the presence of maleimide, and the increase in NADPH production by G6PD was detected by following the absorbance at 340 nm. Protein extracts from control granulocytes, MNCs, and erythrocytes showed a continuous and comparable increase in G6PD-dependent NADPH production, whereas no activity of G6PD was observed in any of the patients' samples (Fig 1).

G6PD defect reduces granulocytic NADPH levels

G6PD activity generates NADPH redox equivalents, which are the substrate for the NADPH oxidase in phagocytes. To confirm an insufficient supply of the phagocytic NADPH oxidase complex with NADPH, we determined NADP⁺ and NADPH concentrations in protein extracts of unstimulated blood

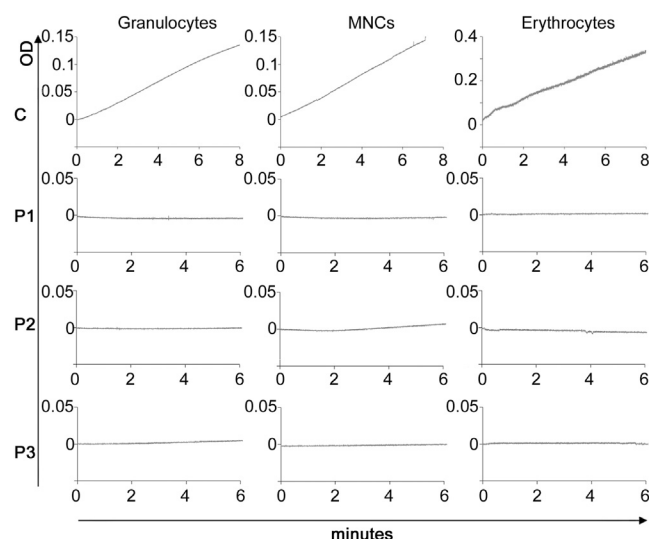


FIG 1. G6PD activity. G6PD activity in MNCs, granulocytes, and erythrocytes (total protein extract: granulocytes/MNCs, 20 μ g; erythrocytes, 200 μ g). The NADP⁺-dependent conversion of glucuronate-6-phosphate to ribulose-5-phosphate by G6PD activity was inhibited in the presence of maleimide, and the increase in NADPH production by G6PD was detected by following absorbance at 340 nm.

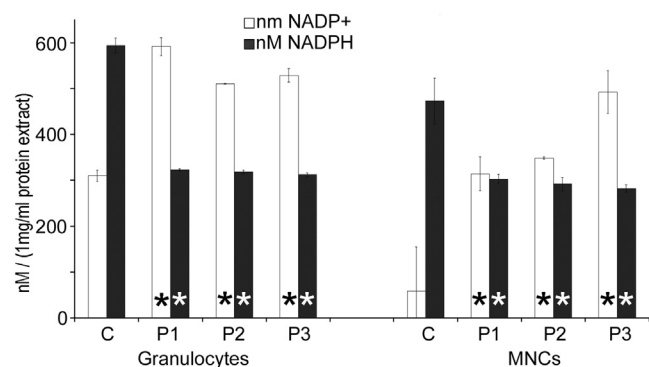


FIG 2. Reduced/oxidized NADP levels. Reduced NADPH and oxidized NADP⁺ concentrations in protein extract normalized to protein content (white and black bars, respectively). Asterisks indicate statistically significant differences ($P < .05$, 2-tailed unpaired *t* test) of concentrations of NADPH and NADP⁺ in granulocytes and MNCs from patients compared with concentrations in corresponding control samples ($n = 8$). C, Healthy control subject.

granulocytes and MNCs normalized to their total protein content (Fig 2).

As expected, in control samples nicotinamide adenine dinucleotide phosphate was primarily found in the reduced state as NADPH (NADPH/NADP⁺ ratio: granulocytes, 1.9; MNCs, 13.6). In granulocytes from G6PD-deficient patients, NADP⁺ levels exceeded NADPH levels. MNCs of P1 contained almost equimolar NADP⁺ and NADPH levels, whereas in MNCs from P2 and P3, NADP⁺ dominated. In all patient samples the differences compared with control NADP⁺ and NADPH levels were statistically significant. This finding strongly indicates that the majority of nicotinamide adenine dinucleotide phosphate in cells from G6PD-deficient patients was oxidized. For human cells, ratios of NADPH/NADP⁺ of 25 in healthy erythrocytes and 0.36 in G6PD-deficient erythrocytes¹⁶ have been reported. This low ratio in G6PD-deficient erythrocytes matches with the low

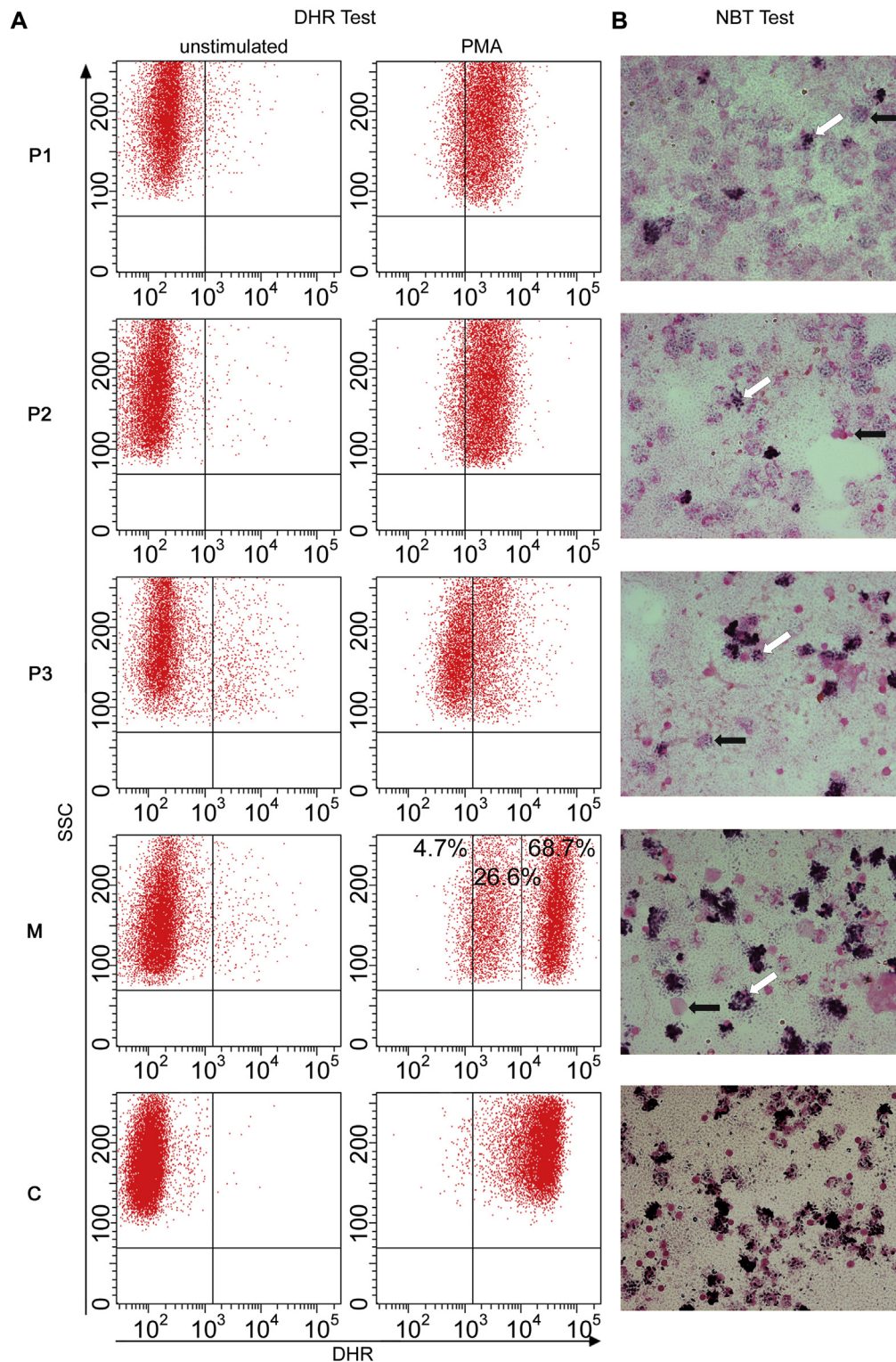


FIG 3. Detection of ROS in blood granulocytes of patients with G6PD deficiency and their carrier mother. Detection of ROS on PMA stimulation by using DHR (A) or NBT (B) assays. White arrows, ROS-positive cells; black arrows, ROS-negative cells. C, Control; M, mother.

ratios we have determined in granulocytes and MNCs of P1 to P3. G6PD-deficient granulocytes contained a background level of reduced NADPH, about half of the control value. This might be

due to G6PD-independent sources of very low levels of reduced NADPH, granting cellular survival but being too low for phagocytic NADPH oxidase function.

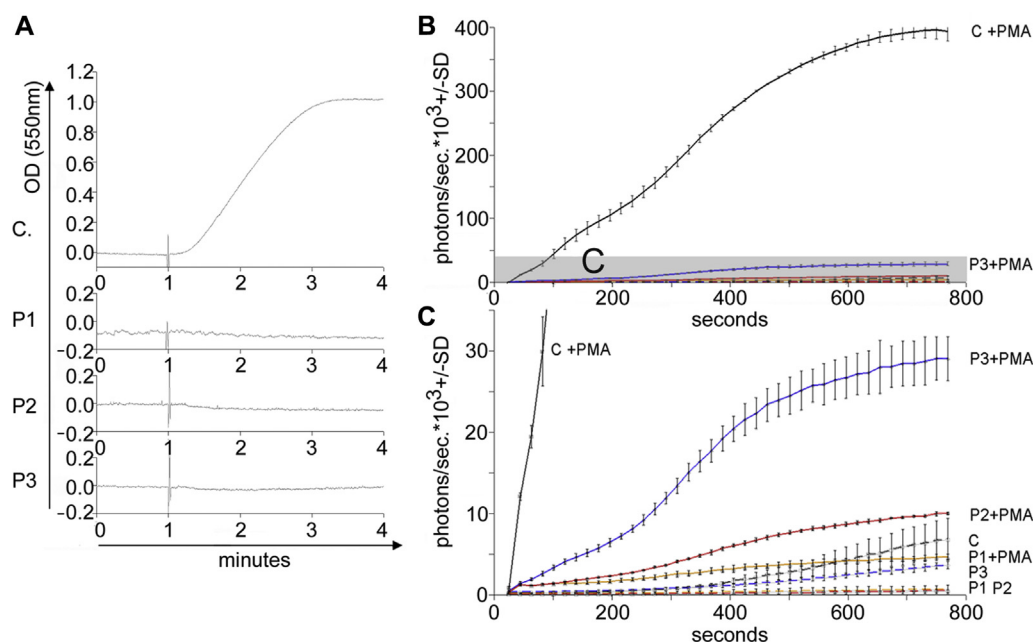


FIG 4. Detection of ROS in blood granulocytes of patients with G6PD deficiency. **A**, ROS quantification by using a cytochrome C assay. **B**, Detection of ROS upon PMA stimulation by using a chemiluminescence-based assay. **C**, Higher magnification of gray-highlighted area in Fig 4, B. C, Control.

NADPH oxidase–dependent ROS formation is impaired in patients with G6PD deficiency

Because G6PD-dependent formation of NADPH is providing the substrate for the phagocyte NADPH oxidase, which in turn leads to production of ROS required for pathogen killing, we next analyzed the NADPH oxidase function in neutrophil granulocytes of the affected brothers and their mother. Like patients with NADPH oxidase–deficient CGD, the 3 brothers analyzed here showed marked reduction in respiratory burst activity in neutrophils on PMA or zymosan stimulation by using DHR or the more sensitive NBT assay, respectively (Fig 3). In addition, the cytochrome C assay showed ROS production of less than the limit of quantification after PMA stimulation (Fig 4, A). However, a more sensitive chemiluminescence assay showed very low residual ROS production on PMA stimulation in the 3 brothers (Fig 4, B and C). X-linked CGD was excluded by means of flow cytometry (fluorescence-activated cell sorting)–based detection of the gp91phox NADPH oxidase subunit on granulocytes (data not shown) and by means of molecular genetic analysis.

In the carrier mother the DHR assay revealed 2 granulocyte populations, one (68.7%) with normal and one with strongly diminished (26.6%) to absent (4.7%) H₂O₂ production. Correspondingly, in the NBT assay the number of cells with intense formazan accumulation also strongly exceeded the number of cells with a weak NBT signal. This phenotype was also observed in carriers of X-linked CGD (Fig 3, A).¹⁷ These data clearly indicate an X-linked genetic disease, possibly caused by either X-linked CGD (*CYBB* gene, X-chromosome Xp21.1), which was excluded by molecular genetic analysis, or by G6PD deficiency (G6PD, X-chromosome Xq28), which was confirmed by means of molecular genetic analysis in this pedigree. Because the family is not consanguineous, autosomal recessive forms of CGD were not analyzed by using molecular genetics.

Absence of NET formation in patients with G6PD deficiency

NADPH oxidase–dependent ROS formation leads to formation of NETs, consisting of chromatin (DNA and histones) decorated with microbicidal proteins, which are an extracellular defense mechanism against a variety of bacterial¹⁸ and fungal⁸ pathogens.

NET formation is impaired in patients with NADPH oxidase–deficient CGD.^{7,8,19} To analyze the functional consequences of insufficient NADPH supply and consequently impaired NADPH oxidase–dependent ROS production in patients with G6PD deficiency, we monitored NET formation in granulocytes from G6PD-deficient patients using fluorescence-based quantification of extracellular DNA release on PMA activation. PMA-stimulated control (n = 24) granulocytes, as well as granulocytes from the mother (3 independent experiments conducted on 3 different days), released chromatin starting 120 minutes after activation. Analysis of these 2 data sets did not reach statistical significance. In granulocyte populations from all 3 patients, no chromatin was released up to 240 minutes after PMA activation (Fig 5, A).

NET formation of neutrophils from the 3 G6PD-deficient patients was indistinguishable from 7 patients with CGD without residual ROS production and from 1 patient with CGD with 100% of neutrophils with faint residual ROS production.¹⁵ However, weak NET formation was observed in a CGD carrier with a chimerism of 18.8% neutrophils with normal ROS production, suggesting an ROS concentration–dependent threshold for induction of NET formation. NET formation differed significantly between the 3 brothers and a group of 24 unrelated healthy control subjects.

NET formation in neutrophils from G6PD-deficient patients could not be induced by LPS, but cells weakly responded to ionomycin, suggesting an NADPH oxidase–independent mechanism of NET formation.²⁰ In patients with CGD and in neutrophils from a patient with G6PD deficiency, the presence of

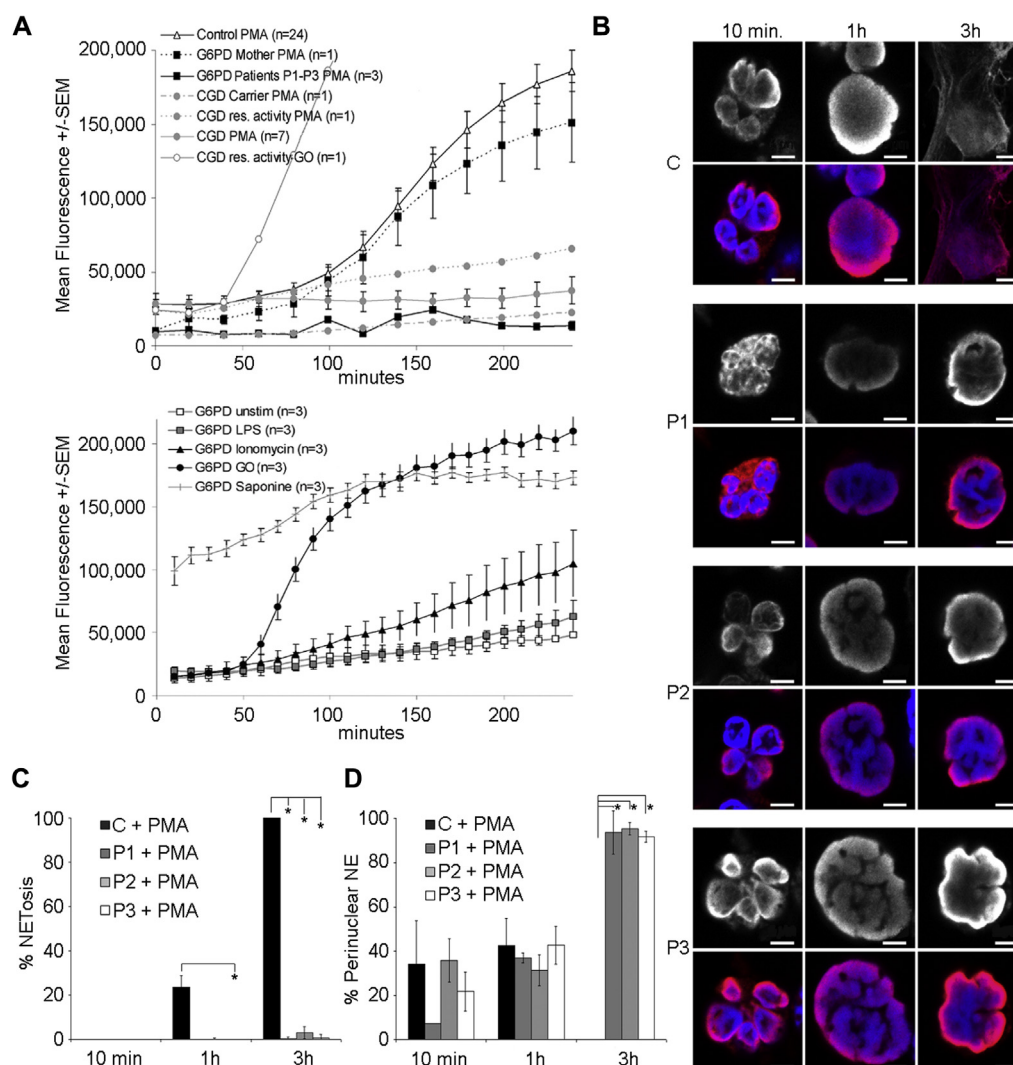


FIG 5. NETosis. **A**, NET kinetics (control subject, G6PD-carrier mother, G6PD deficiency, CGD carrier [18.8% normal and 81.2% cells without ROS], CGD [residual ROS¹⁶], and CGD [without ROS]). GO, Glucose oxidase-treated. **B**, Immunofluorescence (red, NE; blue, 4',6-diamidino-2-phenylindole [DAPI]) of control or G6PD-deficient neutrophils. Black/white, NE; merged: NE/DAPI. **C**, Percentage of NETotic cells (panel B; $n \geq 250$ cells). **D**, Percentage of cells with perinuclear NE signal (panel B; $n \geq 300$ cells).

glucose oxidase as an external source of ROS was able to bypass defective ROS production to restore normal NET formation (Fig 5, A).

Immunofluorescence microscopy for visualization of NETs confirmed these results, showing cellular disintegration and DNA release by control granulocytes at 3 hours after PMA activation and absence of NET release in granulocytes from patients with G6PD deficiency (Fig 5, B). Quantitative analysis of NET formation in granulocytes revealed an increase in the percentage of cells showing macroscopic signs of NETosis 1 and 3 hours after PMA stimulation only in control cells (Fig 5, C). In granulocytes from patients, the percentage of cells showing perinuclear accumulation of neutrophil elastase (NE) increased to greater than 90% within 3 hours of PMA stimulation (Fig 5, D), without showing NETosis (Fig 5, C), as previously observed by our group in patients with CGD,²¹ suggesting an NADPH oxidase-independent process for neutrophil elastase translocation.

DISCUSSION

Patients with G6PD deficiency usually present with hemolytic anemia triggered by oxidative stress induced by drugs, chemicals, or infection.¹ Although G6PD is also expressed in nonerythroid hematologic cells, such as neutrophils, overt predisposition to infection does not seem to be a hallmark of the disease (Table E1 in this article's Online Repository at www.jacionline.org).¹ We show here that severe G6PD deficiency with very low levels of enzyme function leads to alterations in host defense and concomitant susceptibility to infection, mimicking the phagocyte NADPH oxidase defect in patients with CGD. This phenotype adds to the list of other CGD-like syndromes, such as complete myeloperoxidase, Rac2, glutathione peroxidase, glutathione reductase, or glutathione synthetase deficiency.²²⁻²⁵

G6PD is a key enzyme for NADPH-level homeostasis,²⁶ providing NADPH as a substrate for the NADPH oxidase. Therefore severely reduced G6PD enzyme activity in phagocytes

results in impaired reduction of molecular oxygen to superoxide and other ROS required for effective microbial killing.^{27,28} We have previously shown that in patients with CGD, deficient formation of NETs accounts for impaired microbial killing.^{7,8} The 3 brothers with G6PD deficiency (Toledo variant) analyzed here were first given a diagnosis of CGD based on severely impaired production of ROS. We found that G6PD activity was reduced markedly and to the same extent in erythrocytes, MNCs, and neutrophils of these patients. Accordingly, NET formation was completely absent. The complete absence of NET formation in the patients analyzed here is in contrast to a study on G6PD-deficient patients with the Taiwan-Hakka variant, who had normal NET formation.²⁹ However, in those patients about 48% of normal G6PD activity and a 35.9% NADPH/NADP⁺ ratio was registered compared with normal neutrophils. Toledo-type G6PD mutations are associated with class II severe G6PD deficiency,³⁰ which is in line with our observations.

There is likely a threshold of NADPH oxidase-produced ROS required for NET formation because analysis of a CGD carrier with a low percentage of ROS-producing neutrophils showed reduced NET formation. In contrast, the carrier mother of P1 to P3 with a chimerism of 25.1% neutrophils with very low residual production and 70.2% neutrophils with normal H₂O₂ production showed normal NET formation. In her sons the residual amount of ROS production was insufficient for NET formation. G6PD deficiency class I/II with severely impaired enzyme function can thus predispose patients not only to hemolytic crises but also to severe infections with bacteria and fungi. Reported cases are scarce, but document invasive infections, including lymphadenitis, pneumonia, osteomyelitis, gastrointestinal, and cerebral infection, as well as septicemia with gram-positive and gram-negative bacteria (*Acinetobacter baumannii*, *Brucella* species, *Campylobacter jejuni*, *Chromobacterium violaceum*, *Citrobacter freundii*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, meningococci, *Pseudomonas aeruginosa*, *Salmonella* species, *Staphylococcus aureus*, *Staphylococcus epidermidis*, streptococci, and *Stenotrophomonas maltophilia*) and *Aspergillus* species (see Table E1).

Mutations and G6PD enzyme activity were not reported in all of these patients, but in all documented cases enzyme function was very low or absent. Likewise, localization of infections (lymphadenitis, pneumonia, and delayed wound healing) and causative agents (*Serratia marcescens*) in P1 and P2 were also reminiscent of the disease spectrum observed in patients with CGD and prompt discussions about the necessity for carefully selected antibiotic and antimycotic prophylaxis or even hematopoietic stem cell transplantation in patients with severe G6PD deficiency.

In a Dutch patient with severe erythrocyte G6PD deficiency, residual ROS production in 16.3% of control neutrophils (4% residual G6PD enzyme activity) was sufficient for killing of *E coli* *in vitro*.³¹ This is in line with studies in NADPH oxidase-deficient mice, in which 4% to 8% of granulocytes with normal ROS production after bone marrow transplantation were sufficient for pathogen elimination.³² In 24 Israeli patients average G6PD enzyme activity of 13.6% in neutrophils with residual ROS production of 42.1% was sufficient for *E coli* killing and absence of clinical bacterial or fungal infections.³³ NET formation was not analyzed in these patients, but it is likely to be normal. This highlights the importance of determining G6PD enzyme activity, as well as levels of ROS production for optimal counseling of class

I/II G6PD-deficient patients with very low or absent levels of enzyme function. The main weakness of our study is the small number of patients analyzed, and therefore we are currently recruiting other patients with severe G6PD deficiency for immunologic analysis, given the importance of our findings for management of patients with this disease.

Because predisposition to severe infection is likely not familiar to hematologists caring for patients with G6PD deficiency, we propose an escalating diagnostic algorithm, starting with determining the level of G6PD enzyme activity in erythrocytes. To avoid possible unintended consequences of routine screening, such as misdiagnoses, repeated testing, inappropriate therapies, anxiety, and costs in a condition shared by 400 million persons worldwide, further testing should only be done in patients with severely reduced erythrocyte G6PD enzyme activity of less than 10%. We propose that in these patients G6PD activity should be determined also in neutrophils³⁴ because enzyme activities can differ between these cell types.³³ In patients with less than 10% neutrophil enzyme activity, ROS production should be quantified by using a DHR test. If fluorescence intensity is reduced compared with control values, a NET assay should determine functional consequences of the defect as to antimicrobial defense.

In conclusion, we have shown that severely affected patients with G6PD deficiency and absent NET formation can present a phenocopy of CGD and are likely to have an increased susceptibility to invasive bacterial and fungal infection, prompting the decision for appropriate antibiotic and antimycotic prophylaxis. In patients with CGD, disease outcome is dependent on levels of residual ROS production,³⁵ mandating hematopoietic stem cell transplantation as curative treatment in patients with CGD without residual ROS. It certainly remains a matter of debate whether G6PD-deficient patients with very low residual ROS production should also receive curative hematopoietic stem cell transplantation.

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Clinical implications: Severe G6PD deficiency can lead to absence of NET formation and susceptibility to infection. A decision about required antimicrobial prophylaxis can be guided by analysis of ROS production and NET formation.

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TABLE E1. Infections in published patients with severe G6PD deficiency and phagocyte dysfunction

Kindred	Patient ID	Age (y)/sex	Mutation of G6PD	Activity of G6PD (% of control)	DHR/NBT/ROS production/O ₂ consumption (% of control)	Country of origin	Outcome status	Invasive infections with gram-positive bacteria	Invasive infections with gram-negative bacteria	Other	Reference
A	1	34/M	Barcelona	L: 1.4	NBT: 11, O ₂ -production: 8	Spanish	NS	—	—	25-34 y: 3× pneumonia with pleural effusion	Vives Corrons et al ^{E1}
B	2	3.5/M	180-182 delTCT Amsterdam	N: 0	O ₂ consumption: 19.8-10.3	Germany	NS	—	<i>Chromobacterium violaceum</i> pneumonia and osteomyelitis, sepsis	—	van Bruggen et al ^{E2}
	3	15/M		N: 0	O ₂ consumption: 21.3-11.1		NS	—	—	15 y: recurrent fever, recurrent gastroenteritis, disseminated <i>Aspergillus</i> species infection (lung, CNS, soft tissue)	
C	4	52/F	ND	L: 1.7	NBT: negative	United States, white		—	<i>Escherichia coli</i> sepsis <i>Klebsiella pneumoniae</i> pneumonia	Recurrent pneumonia, UTI, cellulitis, splenectomy	Cooper et al ^{E3}
D	5	16/M	ND	ND			NS	—	—	Recurrent infection	Baehner et al ^{E4}
E	6	17/M	ND	L: 0	NBT: <1	Iceland/France/Germany	NS	13-17 y: <i>Staphylococcus aureus</i> 12× cervical lymphadenitis (bilateral)	—	10 y: bronchopneumonia, no pathogen isolated	Gray et al ^{E5}
	7	16/M	ND	0	NBT: <1		NS	—	—	15 y: erythema nodosum 16 y: cervical lymphadenitis	
	8	8/M	ND	0	NBT: <1		NS	—	—	—	
—	9-29	21 neonates	ND	ND	ND	Saudi Arabia	5	8 patients: <i>Staphylococcus epidermidis</i> 2 patients: other infections (not specified) 2 patients: group D and 1 group B streptococci	4 patients: <i>Klebsiella</i> species/ <i>Enterobacter</i> ; 3 patients: <i>E. coli</i> ; 1 patient: <i>H. influenzae</i> ; 2 patients: other infections (not specified)	Catalase-positive sepsis (11 patients: early sepsis, 10 patients: late sepsis)	Abu-Osba et al ^{E6}
F	30	39/F	514C→T	L: 0.5 N: 2.6-5.4	H ₂ O ₂ production: 16%		NS	<i>Streptococcus pneumoniae</i> pneumonia	—	Cystitis, recurrent airway infection	Roos et al ^{E7}
—	31-52	22 adults with sepsis after severe injury	Type A: 202/376	E: 17	DHR: normal	African American	1 (ARDS, septicemia)	3 patients: sepsis (1 patient: G+ and G-) 1 patient: G+ in sputum 4 patients: G+ and G- in sputum	1 patient: G- in sputum	33 patients: bronchial infection, 11 patients: wound infection 22 patients: sepsis	Spolarics et al ^{E8}
G	53	5/M	Nara	N: 0	DHR: 52	Portugal	NS	—	—	4× Gastroenteritis, recurrent upper respiratory tract infection	Costa et al ^{E9}
H	54	3/M	ND	ND	ND		NS	—	<i>Campylobacter jejuni</i> gastroenteritis	—	Mamlok et al ^{E10}
	55	3/M	ND	ND	ND			—	<i>Chromobacterium violaceum</i> sepsis	—	Mamlok et al ^{E11}
—	56-130	75 children, 1-14 y	ND	ND	ND	Saudi Arabia	NS	<i>Streptococcus pneumoniae</i> , α-hemolytic streptococci	<i>Salmonella</i> species, <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Haemophilus influenzae</i> , meningococci	Type of infections (G6PD status not specified): septicemia, meningitis, osteomyelitis, typhoid fever	Mallouh and Abu-Osba ^{E12}

(Continued)

TABLE E1. (Continued)

Kindred	Patient ID	Age (y)/sex	Mutation of G6PD	Activity of G6PD (% of control)	DHR/NBT/ROS production/O ₂ consumption (% of control)	Country of origin	Outcome status	Invasive infections with gram-positive bacteria	Invasive infections with gram-negative bacteria	Other	Reference
I	131	12/M	ND	ND	ND	African American	NS	—	Pneumonia: <i>Stenotrophomonas maltophilia</i> , <i>Pseudomonas aeruginosa</i>	Congenital CMV infection	Harthan and Heger et al ^{E13}
J	132	17/M	ND	ND	ND		NS	—	Brucellosis	—	Pamuk et al ^{E14}
K	133	68/M	ND	ND	ND	Polish	NS	—	Pneumonia: <i>Citrobacter freundii</i>	—	Nowinski et al ^{E15}
L	134	35/M	ND	ND	ND	Taiwan	NS	—	Pneumonia: <i>Acinetobacter baumannii</i>	—	Yang et al ^{E16}
M	135	30/M	ND	ND	ND	African American	NS	Maxillofacial infection: <i>Streptococcus milleri</i> II	—	—	Quereshy et al ^{E17}
N	136	9 mo/M	ND	ND	ND	African American	NS	9 mo, furunculosis: MRSA; 2 y, neck abscess: MRSA	—	1 y, 2 mo and 1 y, 5 mo: skin abscesses; 1 y, 12 months to 1 years, 11 mo: cellulitis, impetigo (no organism documented)	Paek et al ^{E18}

Two premature infants with *Clostridium difficile* enteritis are not shown here^{E19} to exclude bias based on prematurity.

ARDS, Acute respiratory distress syndrome; CNS, central nervous system; E, erythrocytes; G, gram staining; L, leukocytes; MRSA, methicillin-resistant *Staphylococcus aureus*; N, neutrophils; ND, not done; NS, not specified; UTI, urinary tract infection.