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 2017;139:212-9.)

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

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-gluconodelta-lactone 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]

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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 fungi[6-8] 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 (Ethikkomission 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 μmol/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 μmol/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, Regensdorf, 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 μL of this packed erythrocyte cell pellet was diluted in 300 μL 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 μg of total protein or erythrocyte lysates containing 200 μg 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 μL 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 Zychlinsky[13]), washed, and stained with anti-mouse IgG Alexa Fluor 555