

BACTERICIDAL ACTIVITY OF POLYMORPHONUCLEAR NEUTROPHILS IN INDIVIDUALS SEVERELY DEFICIENT IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most prevalent inherited diseases, affecting approximately 400 million individuals worldwide.¹ In the Arab world the incidence varies from 8% to 50%.²⁻⁷ Studies in Bahrain have shown a prevalence of 21%-26%,^{2,8} and have identified six variants of the disease.³

While the effect of G6PD deficiency on red blood cells (RBC) is well known, there is less information available regarding the effect and impact of G6PD deficiency on polymorphonuclear neutrophil (PMN) function and its clinical impact. When PMNs are stimulated, the enzymatic nicotinamide-adenine dinucleotide phosphate (NADP) oxidase system is activated, resulting in the production of free oxygen radicals, which are essential for killing phagocytized microorganisms.⁹ The impairment of this PMN metabolic process leads to the inability of host defenses to kill ingested pathogens. This is well demonstrated in patients with chronic granulomatous disease (CGD) who suffer from recurrent infections.¹⁰ As G6PD is also involved in the regeneration of NADP, which is required for the bactericidal activity of PMNs, it is expected that PMN G6PD deficiency would affect their killing ability, and be clinically associated with recurrent infections.

Literature reports have shown increased frequency of RBC G6PD deficiency in patients with typhoid fever, as compared to the general population,^{11,12} and of increased susceptibility to catalase-positive bacterial infection in RBC G6PD deficient-newborn infants.¹³ Baehner et al.¹⁴ reported a study of 10 patients with varying degrees of PMN G6PD deficiency. Leukocytes from these patients with G6PD levels between 20% and 50% of normals had normal bactericidal activity, and a child with leukocyte G6PD levels of 5% had diminished in vitro bactericidal activity.¹⁴ A single patient with total leukocyte G6PD deficiency and reduced bacterial killing in vitro had a history of recurrent *E. coli* septicemia, and finally died

from *E. coli* and *K. pneumoniae* sepsis.^{14,15}

In this study, the G6PD levels of PMNs isolated from 20 healthy individuals with severe RBC G6PD deficiency and 20 normal controls were determined, and these were correlated with their in vitro bactericidal activity against catalase-positive and catalase-negative microorganisms and their ability to reduce NBT.

Materials and Methods

Blood from 40 adult males—20 controls and 20 with severe erythrocyte G6PD deficiency—attending the blood bank of the Salmaniya Medical Center (SMC) in Bahrain was collected in heparinized tubes. At the time of blood collection all donors were healthy with no history of recurrent or current infections.

Eight mL of the blood was mixed with 4 mL of Hesperan^R (6% hetastarch in saline, Du Pont, UK) and allowed to sediment for 45 minutes at room temperature. The PMNs were separated, as reported previously, with slight modifications.¹⁶ The separated PMNs were washed once with phosphate-buffered-saline (PBS) pH 7.2, and resuspended in 1 mL saline. Total leukocyte count was done using a hemocytometer, and a smear was prepared and stained with Leishman's stain to determine the percentage of the PMNs. The viability of the isolated PMNs was determined using trypan blue.

The G6PD activity of the RBCs was measured according to Ardati et al.³ For the PMN G6PD activity, 500 μ L of the PMN suspension was frozen at -70°C . After thawing, the suspension was centrifuged at 2000 rpm for 5

TABLE 1. RBC and PMN G6PD activity in normal and G6PD-deficient individuals.

G6PD class*	Subjects	No.	G6PD activity	
			RBC u/10 ¹² cells**	PMN u/10 ⁹ cells
IV	Normal	20	119 \pm 29.9	13.2 \pm 2.6
II b	Partially deficient	02	12.35 \pm 0.55	11.2 \pm 0.9
II a	Severely deficient	18	<2.0	4.4 \pm 1.0

*Classification according to Beutler,¹⁹ with proposed modification of Ardati et al.³; **one unit (u) of G6PD is the quantity of enzyme which reduces one μ mol of NADP per minute.

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minutes to remove cellular debris, and the lysate was used for the G6PD assay as in the RBCs.³ Nitroblue tetrazolium dye reduction test was done according to Sigma bulletin 840,¹⁷ using a PMN suspension instead of whole blood.¹⁶

The pellet from the Hetastarch separation was washed twice with heparinized saline (50 u/100 mL saline) and centrifuged at 900 rpm for 5 minutes. Cells (80%-90% PMNs) were resuspended in 0.1% gelatin in Hank's Balanced Salt Solution, pH 7.5 (gelatin-HBSS). PMNs were counted in a hemocytometer and the cell number was adjusted to 1×10^7 PMNs/mL.

Staphylococcus epidermidis and *Streptococcus viridans* were isolates obtained from the microbiology laboratory at SMC. Cultures were prepared from frozen stocks by inoculating a brain heart infusion (BHI) agar plate and incubating at 37°C, overnight. A working bacterial suspension was prepared by inoculating one colony of bacteria from the BHI agar plate in 10 mL of BHI broth and incubating overnight at 37°C. One mL of this culture was centrifuged at 3000 rpm for 15 minutes at room temperature, washed twice with saline and resuspended in 0.1% gelatin-HBSS. The bacterial suspension was standardized to 5.0×10^6 /mL, using a turbidity standard.

The method of Van Furth et al.,¹⁸ with slight modifications, was used for the bactericidal activity of the PMNs. A volume of 0.5 mL of the PMN suspension (5.0×10^6 PMNs) was added to an equal volume of bacterial suspension (2.5×10^6 bacteria) and the mixture incubated for 20 minutes at 37°C in a shaking bath. The process was stopped by immersing the tubes in crushed ice for 1 minute and extracellular bacteria were removed by washing twice with cold gelatin-HBSS and reincubated at 37°C. Samples were taken at intervals, and intracellular bacteria were liberated by lysing the PMNs with sterile cold aqueous solution of 0.01% bovine serum albumin. Bacteria were plated on BHI agar.

G6PD levels were compared using the Student's *t*-test, and the bactericidal activity of the PMNs was analyzed using the geometric mean (GM) of viable counts. The geometric mean was obtained by changing the viable counts to logarithm. The mean and the standard error (SE) of the log values was calculated and the antilog was presented as the $GM \pm SE$.

Results

Our results show that in the erythrocyte G6PD normal group, the enzyme activity in the RBCs was 119 ± 30 u/ 10^{12} cells and in the PMNs, 13.2 ± 2.6 u/ 10^9 cells, while in the deficient group, 18 individuals had no detectable activity (<2 u/ 10^{12} cells) in their RBCs, and their PMN G6PD level was 4.4 ± 1.0 u/ 10^9 PMNs, which is 33% of the controls, while the other two with partial RBC G6PD deficiency (11.8 and 12.9 u/ 10^{12} RBCs) had normal levels of the enzyme in their PMNs (10.3 and 12.1 u/ 10^9 PMNs), respectively (Table 1).

For the comparison of intracellular bactericidal killing of the PMNs, two different species of bacteria were used: *Staphylococcus epidermidis* (catalase positive) and *Streptococcus viridans* (catalase negative). In both normal and G6PD-deficient individuals, the geometric mean of the viable bacterial counts at 60 and 120 minutes following phagocytosis showed no difference for both catalase-positive and catalase-negative organisms (Figure 1). As well, there was no difference between the two groups in the nitroblue tetrazolium reduction test after stimulation of the PMNs.

Discussion

Since the PMN bactericidal activity is dependent on NADP production catalyzed by G6PD, it is reasonable to expect impaired bactericidal activity in individuals with G6PD deficiency. The impaired bactericidal activity is specifically noted with catalase-positive organisms, as they

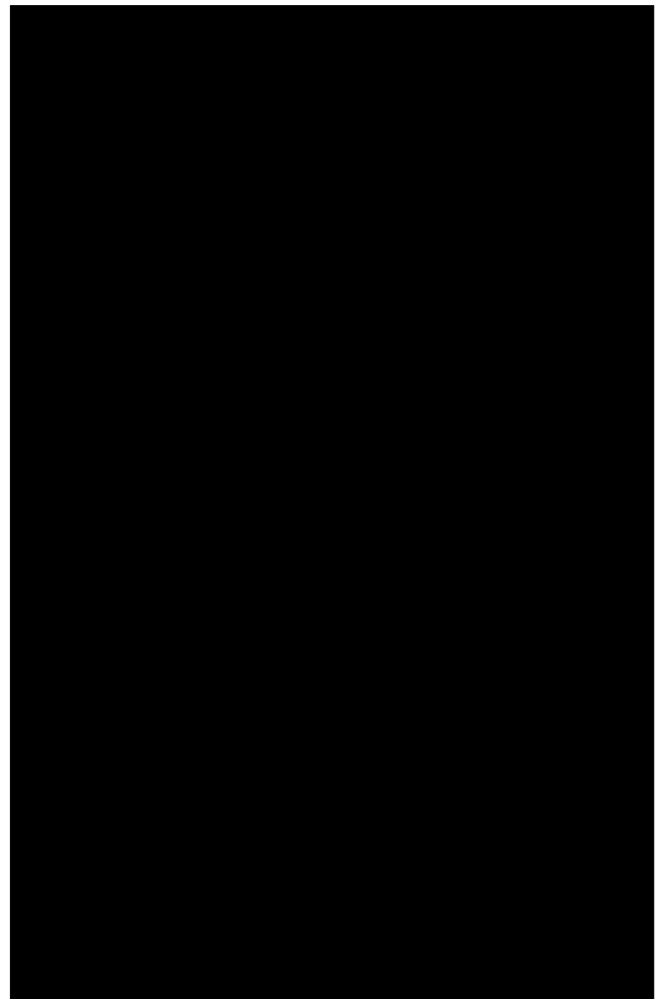


FIGURE 1. The geometric mean of viable counts after the in vitro killing of A) *S. epidermidis* (catalase-positive), and B) *S. viridans* (catalase-negative) by PMNs from normal (—) and G6PD-deficient (.....) individuals. Data shown are the average of 20 individuals for each group in A and 10 in B.

are capable of destroying the H₂O₂ that the phagocytes generate.^{9,10} This is clearly demonstrated in the case of patients with chronic granulomatous disease (CGD), who suffer from recurrent infections due to catalase-positive organisms.^{10,20}

As mentioned above, two organisms, *S. epidermidis* and *S. viridans* were used. There was effective bacterial killing by the PMNs from both normal and G6PD-deficient groups, despite the 33% PMN G6PD activity in the deficient group.

Our data and those of the biochemical assay¹⁶ are in agreement with studies where the PMN G6PD activity was above 5%.²¹⁻²⁴ The few patients with G6PD deficiency and recurrent catalase-positive infections reported in the literature had undetectable leukocyte G6PD activity.^{14,15,25,26}

In an earlier report,³ a proposal to modify the classification of G6PD deficiency according to the enzyme level in RBC was made, distinguishing between complete absence of G6PD in erythrocytes, and severe yet incomplete RBC G6PD deficiency. A subsequent study supported this proposal based on the finding that when erythrocyte G6PD activity is completely absent, the PMN level is significantly lower than the normal control, whereas in individuals with severe (<10% of normal) but incomplete erythrocyte G6PD deficiency, the PMN G6PD level is not different from the normals.¹⁶ It is noted in this study (Table 1) that the two individuals with severe yet incomplete RBC deficiency had PMN levels not different from the normals. This observation further emphasizes the need to distinguish between individuals with near complete absence of RBC G6PD activity, and those with severe yet incomplete G6PD deficiency.

In conclusion, the bactericidal activity of the PMNs is not impaired in individuals with a 33% PMN G6PD activity and a complete G6PD deficiency in their RBCs. The critical level of G6PD activity in the PMN below which the functional bactericidal activity is impaired appears to be around 5% of the normal. Thus, it would be advisable to include a quantitative leukocyte G6PD measurement while clinically evaluating patients with recurrent bacterial infections, particularly if these infections are due to catalase-positive organisms.

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