Hiroshima Journal of Medical Sciences
Vol. 32, No. 2, June, 1983
HIJM 32-27

Functional Analysis of Polymorphonuclear Leukocytes in Siblings of Glucose-6-Phosphate Dehydrogenase Deficiency*)

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(Received March 5, 1983)

Key words: *Glucose-6-phosphate dehydrogenase, Polymorphonuclear leukocytes, Chronic granulomatous disease*

ABSTRACT

Several functions of polymorphonuclear leukocytes (PMNL) were examined in two siblings with glucose-6-phosphate dehydrogenase (G6PD) deficiency. In spite of marked depression of G6PD activity of PMNL, the patients had no susceptibility to bacterial infections. Qualitative nitroblue tetrazolium (NBT) reduction was normal in both cases. Quantitative NBT reduction was normal in one case and subnormal in the other. Bactericidal activity also was normal. However, superoxide anion (O_2^-) release, oxygen (O_2) consumption and chemiluminescence (CL) response were significantly decreased in both cases. This dysfunction was more marked when glucose was added extracellularly. It is concluded that these tests are more suitable than the NBT test for the screening of PMNL G6PD deficiency. It is also considered that there is a large reserve supply of active oxygens necessary for normal bactericidal activity of PMNL.

INTRODUCTION

Total deficiency of PMNL G6PD is a hereditary disorder resembling chronic granulomatous disease (CGD), in which PMNL show defective respiratory burst and decreased bactericidal activity against catalase-positive strains[1,3,6]. A previous report described PMNL with only 25% G6PD activity but normal microbicidal activity and normal respiratory burst[14], suggesting that the normal G6PD reserve is much greater than is absolutely needed for the functional integrity of the hexose monophosphate shunt (HMPS). Since Cooper reported in 1972 that NBT reduction was defective in severe G6PD deficiency[3,4], it has often been used for the screening of this disease. However, the accurate lower limit of activity of this enzyme for normal NBT reduction remains to be determined[1]. Recently O_2^- release and CL response have been utilized as indexes of respiratory burst[2,9]. To our knowledge, these parameters have never been measured in this disease. We had a chance to examine siblings whose PMNL have only 6-7% G6PD activity, and we analysed several functions of PMNL.

MATERIALS AND METHODS

Ferricytochrome C type III, phorbol myristate acetate (PMA), cytochalasin D (cyt. D), concanavalin A (con. A) and zymosan A were purchased from Sigma Chem. Co.

PMNL were isolated from venous blood of the patients and normal volunteers by the method as described previously[8]. Informed written consent was obtained from these subjects. Mononuclear cells (MNC) were separated on a Ficoll-sodium diatrizoate gradient.

G6PD activity: PMNL or MNC were suspended in 0.1 M Tris-HCl (pH. 7.5) with 1 mM

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EDTA. The cells were destroyed by sonicator W225R for 5 min. (at intensity 3) in ice water bath, then centrifuged at 30,000 g for 30 min. The enzyme activity of the supernatant was assayed spectrophotometrically at 340 nm. The reaction mixture contained 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.26 mM NADP and 5 mM G6P.

NBT reduction test: Quantitative NBT testing was done by Okamura's modification of Baehner's method[12]. Qualitative NBT testing was done according to Gifford's method[5]. The percentage of formazan positive cells among the PMNL and MNC which adhered to the cover slip was estimated by light microscopy. In these tests, PMA (100 ng/ml), con. A (100 μg/ml) plus cyt. D (10 μg/ml), or E. coli lipopolysaccharide (100 μg/ml) were added as stimulating agents.

O_2 consumption of PMNL was measured by the O_2 electrode polarographic method previously reported[10]. PMNL were stimulated by 0.5 mg of zymosan opsonized with fresh human serum (OPZ) with stirring.

CL was measured with a luminescence reader (Aloka Co.). Luminol was added for the enhancement of CL. The reaction mixture contained 1×10^6 PMNL, 0.25 mM luminol, 2.5 mg OPZ and 1.0 ml phosphate buffered saline (PBS).

O_2^- release from PMNL was measured by the reduction of ferricytochrome C[2,8]. The reaction mixture contained 5×10^5 PMNL, 0.1 mM cytochrome C and 1 ml PBS: 2.5 mg OPZ, 40 ng PMA, and con. A (100 μg) plus cyt. D (10 μg) were added as stimulating agents. In these three experiments, glucose was added at 2 mM when indicated.

Bactericidal activity of PMNL was determined by a modification of the method of Quie[13]. A bacterial suspension was added to a suspension of PMNL, in a ratio of approximately one bacterium to one PMNL. After incubation for 90 min, viable bacteria were calculated by a pour plate method and expressed as a percentage. In this experiment non-ingested bacteria were not eliminated. To assess the phagocytic activity, 0.1 ml of each incubation mixture was picked up at 30 min and washed three times. The percentage of PMNL which phagocytized bacteria was calculated by light microscopy.

Chemotactic activity of PMNL was examined by an agarose plate method, in a slight modification of Nelson's method[11].

Lysosomal enzyme release (lysozyme, β-glucuronidase) upon phagocytosis of OPZ was estimated by the method reported previously[7].

CASE REPORT

Case 1 and 2 are siblings. A review of the family history showed no other persons with hemolytic anemia. Case 1, T. S., a 4 10/12-year old boy was referred to our hospital in November 1980 for evaluation of an anemia. The patient was the product of a normal pregnancy and delivery. Neonatal hyperbilirubinemia required phototherapy. At 2 4/12 years of age, intermittent abdominal pain, dark urine and icterus appeared. During this episode the patient was not given any drugs or fava beans. Hemolytic anemia was diagnosed and treated. This patient does not have increased susceptibility to bacterial infections. Physical examination on admission showed no particular abnormalities.

Case 2, A. S., the 2 5/12-year-old brother of case 1 was also reffered for the diagnosis of an anemia. The patient grew normally until 1 1/12 year of age, when symptoms similar to those in case 1 appeared. Hemolytic anemia was diagnosed. Two hemolytic crises occurred. Until now, this patient has not shown excessive susceptibility to bacterial infections. Physical examination on admission was normal except for splenomegaly: the spleen was palpable 1 cm below the left costal margin.

Laboratory results were: normal RBC, Hematocrit, Hemoglobin, and reticulocyte count; negative direct and indirect Coombs tests; almost normal serum folate, vitamin B12, ferrokinetics, copper and ceruloplasmin. Bone marrow examination showed a normal myeloid to erythroid ratio and normal morphology. The osmotic fragility of the erythrocytes was normal, even after 24 hours. Autohemolysis was not observed whether glucose was added or not. However, Heinz body formation was increased (89% in case 1, 79% in case 2; normal range 0–28%). Screening tests for abnormal hemoglobinopathies were negative. Their erythrocytes G6PD activity was markedly decreased (Table 1), but all other glycolytic enzymes were normal. The G6PD activity of their parents' erythrocytes was normal. On basis of these results, G6PD deficiency was diagnosed. The

Table 1. G6PD activity of blood components of patients and parents

	RBC	RMNL	MNC
Case 1	0.23 U/gHb	0.33 U/10^6cells	0.12 U/10^6cells
Case 2	0.16	0.47	0.17
Father	8.20	5.07	0.83
Mother	7.80	9.33	0.78
Control	9.45	6.45	0.89

enzyme of these patients was found to be a new variant and named G6PD Hiroshima (report submitted). The characteristics of this variant are: decreased Km for G6P, increased utilization of 2-deoxy-G6P, galactose-6-P and deamino-NADP, heat instability, and biphasic pH optimum.

RESULTS

The activity of G6PD in PMNL and MNC is shown in Table 1. PMNL-G6PD activity was markedly decreased in these siblings, being only 6-7% of the control value. The decrease in the enzyme activity was also noted in MNC.

In spite of the reduction of G6PD activity, the NBT reduction rate of glass adherent cells (PMNL and MNC) was normal in both cases whether the cells were stimulated or not (Table 2). The amount of precipitation of formazan

Table 2. Qualitative and quantitative NBT test

	Qualitative (%)		Quantitative (OD 710 nm)		
	Resting	Endotoxin-activated	Resting	PMA	con. A+ cyt. D
Case 1	56.8	94.1	0.078	0.494	0.755
Case 2	54.1	89.3	0.128	0.611	1.030
Father	39.5	84.8	N.D.[1]	N.D.	N.D.
Mother	37.0	75.5	N.D.	N.D.	N.D.
Control	57.5	92.4	0.123	0.629	1.040

1. N. D., not done

on each cell of the patients was almost the same as on the control cells (data not shown). The quantitative NBT reduction test showed slight depression only in case 1, when PMNL were stimulated by PMA and con. A plus cyt. D, or not.

In order to estimate oxygen metabolism more precisely, other parameters relating to respiratory burst were measured. O_2 consumption and CL response were assayed when PMNL were stimulated by OPZ with constant stirring

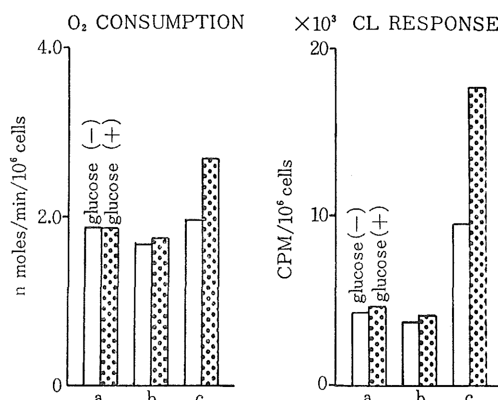

Fig. 1. Left frame shows O_2 consumption of PMNL stimulated by OPZ with constant stirring. Right frame shows CL response of PMNL stimulated by OPZ. Two mM glucose was added (dotted columns) or not added (open columns). A; case 1, b; case 2, c; control. See text for further details.

(Fig. 1). In the absence of glucose, O_2 consumption was normal, while CL response was markedly decreased in both cases. The addition of glucose increased CL response and O_2 consumption in the control PMNL while it did not increase those of G6PD-deficient patients. Therefore, the dysfunction of the patients' PMNL became clear upon the addition of glucose. This tendency was more prominent in the CL response.

O_2^- is the active oxygen which is produced first in PMNL with the acceptance of electron from NADPH. O_2^- release stimulated by OPZ was slightly depressed in the patients' PMNL

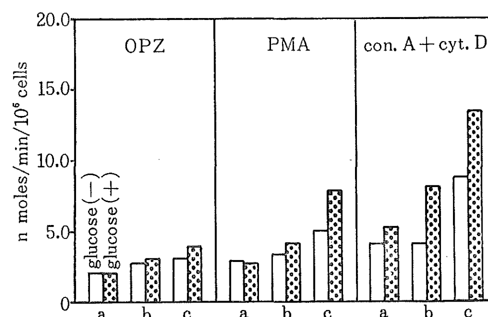

Fig. 2. O_2^- release from PMNL under various conditions. Stimulating agents were OPZ, PMA, and con. A plus cyt. D. The results were compared with (dotted columns) or without (open columns) 2 mM glucose. a; case 1, b; case 2, c; control. See text for further details.

(Fig. 2). The depression of this parameter was more marked when PMA or con. A plus cyt. D was employed as a stimulating agent. This dysfunction was also noted whether glucose was added or not. The addition of glucose markedly enhanced O_2^- release from the control PMNL. In contrast, it did not increase that from the PMNL of case 1 at all. This same tendency was seen in case 2, but less prominently than in case 1.

Bactericidal activity was examined in the presence of extracellular glucose. *Staphylococcus aureus* 209 P, catalase-positive with H_2O_2 production, and *Streptococcus faecalis*, catalase-negative with H_2O_2 production, were employed as the target bacteria. The patients' PMNL killed normally both of these bacteria (Fig. 3); the phagocytic activity was also normal (data not shown).

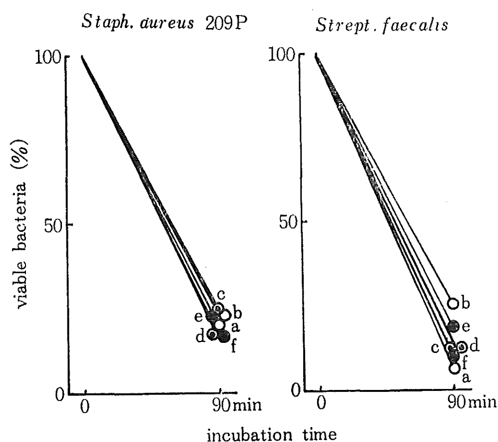

Fig. 3. *In vitro* bactericidal activity of PMNL using *Staphylococcus aureus* 209P and *Streptococcus faecalis*. a; case 1, b; case 2, c; father, d; mother, e; control 1, f; control 2. See text for further details.

Chemotactic activity toward formyl-methionyl-phenylalanine and zymosan-activated serum, chemokinesis in the presence of these chemoattractants, and spontaneous migration were all normal.

Extracellular secretion of lysosomal enzymes was not impaired (data not shown).

DISCUSSION

These patients have intermittent hemolytic crises which were shown to be due to the severe deficiency of G6PD in the erythrocytes. The activity of this enzyme was also depressed in the PMNL and MNC of the patients. G6PD is the first key enzyme of HMPS, which produces NADPH. The depletion of NADPH seen in G6PD-deficient PMNL results in the failure of O_2^- and the following production of active oxygen species. According to previous reports, the range of G6PD deficiency of PMNL which give rise to susceptibility to infections is less than 5% of the control value[1,14]. Baehner showed that PMNL with total deficiency of G6PD showed a marked decrease of H_2O_2 production, NADPH production, bactericidal activity and NBT reduction[1]. It was also shown that marked reduction of H_2O_2, NADPH production and total content of NADPH and NADH were present when G6PD was about 5% of normal value. However, bactericidal activity against *Staphylococcus aureus* was only slightly decreased[1]. In these PMNL, NBT reduction, CL, O_2^- release were not measured.

Although G6PD activity is shown to near by 5% in our patients' PMNL, qualitative NBT reduction was normal. Quantitative NBT reduction was normal in one patient and subnormal in the other. In contrast to the results of NBT reduction tests, CL, O_2^- release and O_2 consumption were decreased. The dysfunction of PMNL in these three tests was more prominent when glucose was added to the incubation medium. Glucose not only supplies G6P which is the substrate of G6PD, but is also necessary for glycolysis. The inclusion in the reaction mixture of glucose, which is known to pass freely through cell membranes of PMNL, raises the intracellular G6P concentration. The enhancing effect of glucose seen in normal PMNL seems to be derived from enhanced HMPS activity, because this enhancement was hardly seen in G6PD-deficient PMNL. In the experiment of O_2^- release the dysfunction was more prominent when PMA and con. A plus cyt. D were used than when OPZ was used. In addition to the effect of glucose, the use of more intense stimulating agents is suitable for the detection of G6PD deficiency.

The dissociation between NBT reduction and the other three parameters which were markedly reduced is unclear. NBT reduction is about half due to O_2^- [2], but that of the other are more closely related to O_2^-. In addition, NBT itself is noxious to PMNL[15]. Thus, NBT reduction test is likely to be more insensitive.

It can be considered that the range of PMNL G6PD which gives rise to defective NBT reduction is less than that which damages other functions. So, reduction of the latter may well reflect a decrease of the NADPH pool and these are more appropriate tests for screening PMNL-G6PD dificiency.

In spite of the disturbed oxygen metabolism described above, bactericidal activity against both catalase-positive and catalase-negative strains was normal. In carriers of CGD with X-linked recessive transmission, about half of these PMNL are defective and resemble the PMNL of CGD patients. In our patients with G6PD deficiency, all PMNL seemed to be equal because almost all PMNL contained formazan deposits. They are able to produce the minimum amount of H_2O_2 necessary to kill catalase-positive strains. This indicates that a very small amount of H_2O_2 is sufficient for the oxidative killing system. In addition to the large reserve of oxygen metabolites for microbial killing may enable these patients to resist bacterial infections. Although we could not find any microbicidal defect in our patients *in vitro* or *in vivo,* the condition which induce hemolytic anemia may give rise to transient dysfunction of PMNL. These patients must be observed carefully in the future.

The exact nature of PMNL G6PD could not be determined in our patients, but it is considered to be the same as erythrocyte G6PD[16]. It may be that the range of G6PD which is necessary for normal resipiratory burst and bacterial killing differs with the type of variant. Further data must be accumulated to confirm this hypothesis.

CONCLUSIONS

O_2^- release, O_2 consumption and CL response of PMNL were decreased in G6PD-deficient siblings in spite of normal NBT reduction. These tests are more suitable than the NBT test for the screening of G6PD deficiency of PMNL.

ACKNOWLEDGMENT

The authers are greatly indebted to Dr. Alice S. Cary for assistance in preparing this manuscript. This work has been supported in part by Grants-in-Aid for Scientific Research (Project Nos. 544050, 448227) from the Ministry of Education, Scientific and Culture of Japan and a Grant-in-Aid from the Japan Medical Research Foundation.

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