Familial Deficiency of Glutathione Reductase in Human Blood Cells

By Hans Loos, Dirk Roos, Ron Weening, and Jaap Houwerzijl

A virtually complete absence of glutathione reductase activity was found in the erythrocytes of all three children (one male, two females) from a consanguineous marriage. Intermediate values were found in the erythrocytes of both parents. The enzyme activity could not be restored either by addition of FAD in vitro or by administration of riboflavin in vivo. The amount of reduced glutathione in the erythrocytes was normal in each case. Severely diminished glutathione stability during incubation with acetylphenylhydrazine was observed in the erythrocytes of the siblings, as well as intermediate stability in the parents' red cells. Clinically, this deficiency was manifested by hemolytic crises after eating fava beans in the eldest daughter (patient), and possibly by cataracts in her own and in her brother's eyes. Very low activities of glutathione reductase were also found in the leukocytes of this family: 13%–15% of normal values for the children and 64%–66% for the parents. Moreover, the same deficiency was found in the purified white blood cells of the propositus: 8% of normal values in the polymorphonuclear (PMN) cells, 4% in the lymphocytes, and 15% in the monocytes, together with 11% in the platelets. Finally, we found an abnormal oxygen consumption of the propositus' PMNs after phagocytosis of zymosan particles, suggesting that the glutathione reductase reaction was involved in the bactericidal capacity of these cells.

GLUTATHIONE REDUCTASE (NAD(P)H: oxidized glutathione oxidoreductase, EC 1.6.4.2) catalyzes the reaction GSSG + NADPH + H+ ⇌ 2 GSH + NADP+. The human erythrocyte enzyme can also utilize NADH as a substrate and consists of a flavoprotein with FAD as the prosthetic group. Partial deficiencies of this enzyme in erythrocytes have been previously described, virtually all of them, however, apparently due to defects in riboflavin intake.

In the present paper, the clinical and biochemical findings in a family with three cases of complete GSSG reductase deficiency in the erythrocytes, unaffected by FAD in vitro or riboflavin in vivo, are described. Markedly depressed activity of this enzyme has also been found in the leukocytes. Both features, as far as we know, have not been described before. Moreover, a high susceptibility of the erythrocytes to oxidative stress in vivo and in vitro is ap-

Abbreviations used in text: GSH, reduced glutathione; GSSG, oxidized glutathione; PMN, polymorphonuclear leukocytes; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; PK, pyruvate kinase; FAD, flavin adenine dinucleotide; HMP shunt, hexose monophosphate shunt.

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Preliminary results indicate that abnormal granulocyte function may also be attributable to the enzyme deficiency.

CASE REPORT

A 22-yr-old woman had complained of tiredness, dizziness, nausea, and vomiting for 2 days, and on the day of admission to hospital noticed that her urine was red. She had experienced the same symptoms to a lesser degree several times during the previous 2 yr. One day before the onset of her illness she had eaten a meal containing a large quantity of fava beans.

The patient was one of three siblings from a consanguineous marriage. The family pedigree is shown in Fig. 1. Her sister and her parents were in good health, but her brother suffered from juvenile cataracts. Neither her brother nor her sister had ever experienced any of her other symptoms, nor had either shown a predisposition toward bacterial diseases. The patient, however, believed that she suffered from infections for somewhat longer than others. Quite recently it was discovered that the patient had also developed bilateral cataracts and that her sister had presented with an undiagnosed eye problem.

Physical examination revealed a pale, white woman with slightly icteric sclerae but no other abnormalities. On admission, she had a hematocrit of 28% and a hemoglobin of 8.7 g/100 ml. The white cell count was 16,200/cu mm, with 92% neutrophils, 6% lymphocytes, and 2% monocytes. The platelet count was 215,000/cu mm; the reticulocytes 6.0%. Several red blood cells showed punctate basophilic stippling; Heinz bodies were not observed. The indirect bilirubin was 3.27 mg/100 ml serum, and the haptoglobin 59 mg/100 ml serum (normal range 100-300 mg/100 ml). The direct Coombs test was negative, and red cell osmotic fragility was normal. Bone marrow examination showed increased erythropoiesis. The urine contained a trace of hemoglobin.

Hemolytic anemia, possibly due to eating fava beans, was proposed. One day after admission her general condition suddenly deteriorated. The hematocrit fell to 21% and blood transfusion was necessary. Thereafter, she quickly recovered and was discharged within 1 wk.

Several months later general hematologic investigations were repeated. The hemoglobin content and number of reticulocytes were normal. The haptoglobin content of the serum was also normal, and the direct antiglobulin test was again negative. The agglutinability of her erythrocytes with two different anti-I and two different anti-i sera, as well as the ability to hemolyze with anti-I serum were normal. Erythrocyte acetylcholinesterase activity was normal, and the acid Ham and sugar water tests were negative. Moreover, pyruvate kinase and glucose-6-phosphate dehydrogenase (G6PD) activities, which in our experience have been found to be consistently elevated during chronic hemolytic anemia, were found to be normal (see Results). Thus, there was no indication for chronic hemolytic anemia after the crisis.

In order to exclude any possible defect in erythrocyte glycolysis, ATP + ADP content, as well as the 2,3-diphosphoglycerate level of the patient’s red cells, were measured. The ATP + ADP level was 1.5 µmole/ml red cells and the 2,3-diphosphoglycerate content 4.9 µmoles/ml, both within the normal ranges.

The assumption that the hemolytic anemia was precipitated by the consumption of fava beans was supported by the fact that the patient had only started eating this vegetable since her marriage 2 yr before. The other members of her family found fava beans unpalatable.

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**Fig. 1.** Pedigree of family M. , Erythrocyte and leukocyte GSSG reductase below normal; , erythrocyte GSSG reductase undetectable, leukocyte GSSG reductase 13%-15% of normal; arrow indicates propositus.
With respect to the cataract, erythrocyte galactose-1-phosphate uridylyl transferase activity was normal.

MATERIALS AND METHODS

Erythrocytes were obtained from blood anticoagulated with acid-citrate-dextrose (U.S.P. formula A) by centrifugation at 2000 g for 10 min at room temperature, followed by aspiration of the plasma and buffy coat. The cells were then washed three times with centrifugation with approximately three volumes of 0.154 M saline.

Leukocytes were prepared from ACD blood stored overnight by a method previously described.4

Granulocytes and lymphocytes were purified from fresh, defibrinated blood after Ficoll-Isopaque centrifugation as previously described.4,5

Monocytes were prepared from mononuclear leukocytes on a Ficoll-Isopaque discontinuous density gradient as described elsewhere.6

Platelet concentrates were prepared from fresh ACD blood by centrifugation at 1000 g for 5 min at room temperature, followed by acidification (10% v/v with ACD) and centrifugation (2200 g for 20 min at room temperature) of the supernatant. The leukocyte and platelet concentrations were determined with a Coulter Counter, model ZF (leukocytes: 100-μm orifice, platelets: 50-μm orifice). All assays were performed simultaneously with patient cells and control cells which were purified in exactly the same way.

Glutathione reductase activity was measured spectrophotometrically at 340 nm by the rate of oxidation of NADPH in the presence of GSSG (Racker)7. For the erythrocyte assay, 0.006 ml of packed red cells was added to 3.0 ml of a reaction mixture containing 41 mM Tris-HCl (pH 7.5 at 25°C), 14 mM MgCl2,* 5.7 mM EDTA, 68 mM KCl, 0.017%, saponin, 1.3 mM GSSG (Boehringer), and 0.1 mM NADPH (Boehringer). The reaction was measured for at least 10 min at 25°C against a blank from which GSSG and NADPH were omitted. Blanks from which either of these substrates were omitted gave the same result. Afterward, the hemoglobin concentration in the cuvette was determined at 540 nm, and the reaction rate calculated as -Δμmoles NADPH/min/g Hb. For the determination of the glutathione reductase activity in leukocytes, granulocytes, monocytes, and lymphocytes, 0.6 ml of cell suspension was added to 2.4 ml of the above reaction mixture. The glutathione reductase activity in the platelets was tested by adding 0.2 ml GSSG solution and 0.2 ml NADPH solution to 2.6 ml of sonicated platelets in the assay buffer, giving the same final concentrations as with the other cells (sonication: 15 sec at 20 kc/sec, 45 W).

Glucose-6-phosphate dehydrogenase activity was determined using a modified Kornberg and Horecker method,6 6-phospho-glucuronate dehydrogenase (6PGD) essentially as described by Glock and McLean,9 and pyruvate kinase (PK) by a modification of the assay published by Tanaka et al.10 Glutathione peroxidase activity was measured according to Paglia and Valentine.11 Reduced glutathione (GSH) was determined by a modification of Stevenson's method with bis(p-nitrophenyl) disulfide reagent.12 After incubation of red cells for 2 hr with acetylphenylhydrazine (final concentration 5 mg/ml in Krebs-Ringer buffer with 17.5 mM glucose), the GSH determination was repeated and compared with the level observed originally, according to Beutler.13 The oxygen consumption of phagocytosing granulocytes was measured in an oxygraph with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, Model No. YSI 5331) as described before.4

RESULTS

The hemolytic crisis provoked by the ingestion of fava beans prompted the determination of glutathione stability, as well as the activities of the enzymes involved in the glutathione redox system in the erythrocytes of members of the patient's family. The results are presented in Table 1. The activities of G6PD, 6PGD, PK, and glutathione peroxidase were all within the normal range. Glutathione reductase activity, however, was undetectable (<0.2 μmoles

*The same buffer solution, containing the required ions, EDTA, and a lytic agent, was used for the G6PD, 6PGD, PK, and GSSG reductase assays in erythrocytes.
Table 1. Biochemical Determinations in the Red Cells of Family M

<table>
<thead>
<tr>
<th>Subjects</th>
<th>GSH* Before</th>
<th>GSH* After</th>
<th>G6PD†</th>
<th>G6PD†</th>
<th>GSSG† Red.</th>
<th>GSSG† Perox.</th>
<th>PK†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>57</td>
<td>23</td>
<td>4.8</td>
<td>2.4</td>
<td>1.7</td>
<td>21</td>
<td>4.2</td>
</tr>
<tr>
<td>Mother</td>
<td>63</td>
<td>32</td>
<td>5.2</td>
<td>2.6</td>
<td>1.7</td>
<td>21</td>
<td>8.4</td>
</tr>
<tr>
<td>Brother</td>
<td>59</td>
<td>6</td>
<td>5.1</td>
<td>2.5</td>
<td>&lt;0.2</td>
<td>21</td>
<td>6.9</td>
</tr>
<tr>
<td>Patient</td>
<td>59</td>
<td>7</td>
<td>5.8</td>
<td>2.7</td>
<td>&lt;0.2</td>
<td>21</td>
<td>7.8</td>
</tr>
<tr>
<td>Sister</td>
<td>56</td>
<td>7</td>
<td>5.2</td>
<td>2.4</td>
<td>&lt;0.2</td>
<td>21</td>
<td>6.8</td>
</tr>
<tr>
<td>25 normals</td>
<td>50-90</td>
<td>&gt; 40</td>
<td>4.8-7.3</td>
<td>1.6-3.2</td>
<td>2.4-4.8</td>
<td>19-48</td>
<td>4.8-9.7</td>
</tr>
</tbody>
</table>

*Reduced glutathione (mg/100 ml packed erythrocytes) before and after incubation for 2 hr at 37°C with acetophenylhydrazine (5 mg/ml blood).
†In μmoles/min/g hemoglobin at 25°C.

NADPH consumed/min/g Hb) in the erythrocytes of the three children. A mixing experiment with normal erythrocytes and those of the patient showed that no inhibitor of the GSSG reductase reaction was present in the latter cells. Incubation of the patient’s red cell lysate with GSSG and NADPH for 2 hr did not reveal GSSG reductase activity. Utilization of 1.7 μmoles NADPH/min/g Hb was found in the glutathione reductase assay of the parent’s erythrocytes; this was well below the normal range (Table 1).

The addition of 1 μM flavin adenine dinucleotide (FAD) prior to the addition of GSSG and NADPH did not increase glutathione reductase activity in lysates of erythrocytes from the family members. In a control sample tested in the same run, glutathione reductase activity increased slightly from 3.3 to 3.9 μmoles GSSG/min/g Hb at 25°C (Table 2). Theoretically, diminished binding of FAD to GSSG reductase may result in suboptimal restoration of activity. Variation of the FAD concentration between 1 and 1000 μM in the GSSG reductase assay of the patient’s red cells, however, did not increase activity. In a control sample tested at the same time, glutathione reductase activity rose from 3.9 to 5.2 μmoles GSSG/min/g Hb upon the addition of FAD to a final concentration of 5 μM prior to the addition of GSSG and NADPH. Finally, the administration of riboflavin in vivo (5 mg daily for 3 days) did not result in any increase of the GSSG reductase in the patient’s erythrocytes.

Table 2. Effect of FAD and Riboflavin on GSSG Reductase Activity in the Erythrocytes of Family M

<table>
<thead>
<tr>
<th>Subjects</th>
<th>GSSG Reductase Activity†</th>
<th>Riboflavin In Vivo (5 mg/day, 3 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FAD In Vitro (1 μM)</td>
</tr>
<tr>
<td>Father</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Mother</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Brother</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Patient</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Sister</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Control</td>
<td>3.5</td>
<td>3.9</td>
</tr>
</tbody>
</table>

†In μmoles/min/g hemoglobin at 25°C.
N.T., Not tested.
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Fig. 2. GSSG reductase activity in concentrated erythrocyte lysate after preincubation with 1 μM FAD. o-o, GSH production in the control erythrocyte lysate; e-e, GSH production in the lysate of the patient's erythrocytes. After preincubation of the lysate in the presence of 1 μM FAD, the reaction was started by the addition of GSSG, NADP, and glucose-6-phosphate. At the time indicated, the GSH produced was estimated as described in the text. The intrinsic G6PD activity of the incubation mixture was about 0.3 IU/ml.

In order to define more clearly the actual glutathione reductase activity of the patient's erythrocytes, this enzyme was measured in a concentrated lysate (approximately 0.06 g Hb/ml). Washed erythrocytes, both from the patient and from a control, were lysed by freezing and thawing three times and were then preincubated with 1 μM FAD at 25°C for 10 min at pH 7.4. Thereafter, GSSG (10 mM), glucose-6-phosphate (20 mM), and NADP+ (1 mM) were added to this mixture, which also contained 17 mM Tris-HCl buffer, 6 mM MgCl2, 2 mM EDTA, 28 mM KCl, and 7 mg/100 ml saponin. The intrinsic G6PD activity in this mixture was approximately 0.3 IU/ml, sufficient to supply the necessary NADPH. After different time intervals, protein-free extracts were prepared and the GSH produced was estimated. The result is presented in Fig. 2. A method of least-squares calculation showed that this method permitted the detection of 0.8% of the GSH production found with the control lysate after 60 min. However, no GSH production was found in the lysate of the patient's erythrocytes.

Replacing NADPH by NADH in the spectrophotometric assay for glutathione reductase of the erythrocytes of all three children did not augment the activity.

In accordance with these findings, we noted decreased GSH stability in the erythrocytes of all members of the family during incubation with acetylphenylhydrazine. Again, the instability was more marked in the erythrocytes of the patient (Fig. 3) and her brother and sister than in the parents (see Table 1). Finally, the Heinz body test, according to Beutler, showed 91% positive cells in the erythrocytes of the patient (control: 26%).

Glucaric acid, a normal constituent of human urine, is found in diminished amounts in G6PD-deficient patients with favism. However, a normal amount of this product was found in the patient's urine as measured by inhibition of granulocytic β-glucuronidase with the method of Marsh (ten normals—24.3%, inhibition by 40% (v/v) urine, range 16.4%–33.5%, no difference between male and female; patient—32.1% inhibition).

Leukocytes isolated from blood stored at 4°C overnight also showed defective glutathione reductase activity in comparison with control values de-
Fig. 3. GSH stability of GSSG reductase-deficient erythrocytes. GSH content of the control erythrocytes; GSH content of the erythrocytes of the patient. Red cells incubated in Ringer solution containing 17.5 mM glucose and 5 mg/ml acetylphenylhydrazine at 37°C and pH 7.4 (hematocrit, 33%). GSH was estimated in protein-free extracts with bis (p-nitro-phenyl) disulfide time (min) according to Prins et al.12

rived from both fresh blood and from stored blood (Table 3). Leukocytes from the children showed about 15% of the normal values; the parents' cells showed about 65%. In order to determine whether or not the leukocyte activity was restricted to a specific cell type, lymphocytes, granulocytes, monocytes, and platelets were purified from the patient's fresh blood. The defect was found to be present in all four cell types (Table 4).

Finally, since it is known that glutathione metabolism is involved in the stimulation of the oxidative metabolism of PMNs after phagocytosis of particles,20 the oxygen consumption of the patient's granulocytes was measured several times. The patient's cells showed only a very brief respiratory burst after challenge with opsonized zymosan particles, corresponding to 13 nmoles O2 per 10⁶ granulocytes (Fig. 4).

The glutathione reductase deficiency in the patient's lymphocytes did not impair the response to mitogens or antigens, or in the mixed lymphocyte reaction (neither stimulating nor responding capacity) as judged by ³H-thymidine incorporation (not shown here). The response of the platelets from all three children to hypotonic stress21 was normal. These findings suggested that glutathione reductase activity was neither required for immune competence of lymphocytes nor for the viability of thrombocytes.

DISCUSSION

To our knowledge, this is the first report of a virtually complete absence of glutathione reductase activity in red blood cells, although a value of 9% of normal has been reported.1 It should be mentioned, however, that this low value has been found in the absence of added FAD and is therefore probably difficult to compare with the values obtained in the current study. The normal levels of GSH in the erythrocytes of the family described here, however, suggest that either the extremely low resting activity of GSSG reductase is sufficient for the maintenance of GSH levels under normal conditions, or that the synthesis of GSH from its constituent amino acids can keep up with its oxidation in vivo, in
Table 3. Glutathione Reductase Activity in the Leukocytes of Family M
(Isolated From ACD Blood Stored Overnight)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>GSSG Red. *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>178</td>
</tr>
<tr>
<td>Mother</td>
<td>187</td>
</tr>
<tr>
<td>Brother</td>
<td>38</td>
</tr>
<tr>
<td>Patient</td>
<td>41</td>
</tr>
<tr>
<td>Sister</td>
<td>37</td>
</tr>
<tr>
<td>10 normals (blood stored overnight)</td>
<td>205–353</td>
</tr>
<tr>
<td>10 normals (fresh blood)</td>
<td>255–330</td>
</tr>
</tbody>
</table>

*In μmoles/min/10¹¹ cells at 25°C.

Table 4. Glutathione Reductase Activity in the Purified Lymphocytes,
Granulocytes, Monocytes, and Platelets of the Patient in Family M

<table>
<thead>
<tr>
<th>Blood Cells of Patient</th>
<th>GSSG Red. *</th>
<th>10 Normals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes†</td>
<td>8</td>
<td>167–266</td>
</tr>
<tr>
<td>Granulocytes†</td>
<td>23</td>
<td>189–350</td>
</tr>
<tr>
<td>Monocytes†</td>
<td>17</td>
<td>84–189</td>
</tr>
<tr>
<td>Platelets‡</td>
<td>0.5</td>
<td>2.3–6.0</td>
</tr>
</tbody>
</table>

*In μmoles/min/10¹¹ cells at 25°C.
†Isolated from fresh defibrinated blood.
‡Isolated from fresh ACD blood.

Fig. 4. Oxygen consumption of GSSG reductase-deficient PMNs. At the time indicated by the arrows, 60 μl opsonized zymosan suspension was added to 600 μl PMN (4 × 10⁶ cells/ml) and the oxygen concentration was measured in an oxygraph with a Clark-type electrode.
which case excess GSSG might be excreted by the erythrocytes. Compensation by the NADH-dependent GSSG reductase is impossible since both NADPH and NADH-dependent enzymes have been found to be deficient in this family. The findings are compatible with the hypothesis that both activities are catalyzed by one molecule.

In the great majority of cases of glutathione reductase deficiency the activity of the enzyme could be restored by addition of FAD in vitro or by administration of riboflavin in vivo. In the present investigation, however, we did not find restoration of activity when FAD was added to the red cell glutathione reductase assay, or when the patient’s erythrocytes were reexamined after 3 days of a riboflavin-rich diet (5 mg/day). It seemed highly unlikely, therefore, that a riboflavin deficiency was the cause of the GSSG reductase defect in this case. The glutathione instability of the erythrocytes as well as the clinical findings before, during, and after the crisis suggested that the patient’s erythrocytes were susceptible to the digestive products of fava beans. This observation was an additional indication that the enzyme defect was different from the nutritionally induced glutathione reductase deficiencies described in the literature.

The fact that the deficiency is found in all blood cell types is to be expected since GSSG reductase consists of two identical subunits. Although some GSSG reductase activity has been found in total leukocytes, lymphocytes, monocytes, and platelets, it seems very probable that one genetic defect is responsible for all the deficiencies observed. The fact that the residual GSSG reductase activity could only be detected in leukocytes and platelets but not in erythrocytes may be due to the continuous synthesis of an unstable enzyme in the nucleated cells and to the short lifespan of platelets. Other investigators have found only partial deficiencies, different pH optima or variable $K_m$ (GSSG) in leukocytes and platelets. The mode of inheritance (Fig. 1) is compatible with an autosomal trait.

The normal amount of glucaric acid in the patient’s urine indicates that an additional defect in glucuronic acid metabolism is no prerequisite for the development of favism, as postulated by Cassimos et al. for G6PD deficiency.

The fact that both the patient and her brother are developing a cataract, while their sister is also complaining about eye troubles, points to a possible link between GSSG reductase deficiency and the occurrence of cataracts. The role of GSH in the eye lens has not yet been fully determined, although the level of this compound and the activities of glutathione reductase and the hexose monophosphate (HMP) shunt in lens cells are high. Possibly, GSH functions by maintaining protein thiol groups in the lens in the reduced state. This thesis would explain the high incidence of cataracts in the family, since oxidation of protein thiol groups cannot be reversed in this case by the combined action of glutathione, glutathione reductase, and the HMP shunt. This reasoning also applies to the cataracts in the eyes of a patient with G6PD deficiency in the leukocytes.

Finally, it seems extremely important to study in more detail the functional capacity of the granulocytes in this family. Quie et al. and Holmes et al. have reported two patients with a hereditary leukocyte glutathione peroxidase de-
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...ficiency; the patients suffered from severe, recurrent infections. The leukocytes of these patients were found to have deficient oxygen consumption and glucose-1-14C oxidation, and they had diminished bacterial killing potential even though they showed normal phagocytosis. Moreover, G6PD deficiency in leukocytes has also been found to induce defective bactericidal activity; these granulocytes were able to phagocytose, but subsequent stimulation of oxygen consumption and H2O2 production were missing.

Our patient’s PMNs also showed an abnormal response to phagocytosable particles. After incubation of the patient’s granulocytes with opsonized zymosan particles, electron microscopy revealed that all cells had taken up these particles. The very limited oxygen consumption by these cells confirmed that reduced glutathione may be involved in the metabolism of hydrogen peroxide. Moreover, the fact that patients with a deficiency of glutathione peroxidase suffer from severe infections, while those with a deficiency of glutathione reductase do not, suggests that the difference in peroxidation plays a crucial role. Possibly, the detoxification of H2O2 by GSH in the glutathione peroxidase reaction is essential for the survival of the cells during phagocytosis. This reaction can still take place in glutathione reductase-deficient cells, albeit to a limited extent. The sudden drop in oxygen consumption of the patient’s granulocytes may thus be caused either by irreversible damage from H2O2 (or a precursor such as O2-), or by lack of GSH as a substrate for production of O2- or H2O2.

ACKNOWLEDGMENT

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