Changes in the Levels of Glutathione in Phagocytosing Human Neutrophils

By Alwin A. Voetman, Johannes A. Loos, and Dirk Roos

The tripeptide γ-glutamyl-cysteinyl-glycine, or glutathione, protects cells against oxidative damage by its ability to react with free radicals and peroxides. From studies with human neutrophilic granulocytes deficient in glutathione metabolism, it is known that the protection by this compound is essential for the proper functioning of these cells. Previous studies have yielded conflicting estimates of reduced glutathione (GSH) and oxidized glutathione (GSSG) in human neutrophils. We have optimized three independent assays to measure glutathione in about 10⁷ human neutrophils. With these methods we have measured the level of GSH and GSSG at rest, during phagocytosis, and during incubation of the cells with a hydrogen-peroxide-generating system. GSH was measured as a rate-limiting cofactor in the formaldehyde dehydrogenase reaction, in the nonspecific colorimetric reaction with 5,5’-dithiobis-(2-nitrobenzoic acid) after cell extraction with sulfuric acid, and in the latter reaction coupled to the glutathione reductase reaction. The first two methods gave falsely high values for GSH; other sulfhydryl compounds probably contributed to the result. With the third method, which measures the sum of GSH plus GSSG, 14.2 ± 2.2 nmole GSH + GSSG/10⁷ neutrophils (mean ± SD, n = 21) were found. GSSG was measured in the glutathione-reductase-coupled reaction after binding GSH to N-ethylmaleimide, followed by separation of GSSG from free N-ethylmaleimide over Sephadex G-10. A level of 0.6 ± 0.8 nmole/10⁷ neutrophils (mean ± SD, n = 12) was found. Subtraction of this value from the value for GSH + GSSG leads to an estimation of about 13 nmole GSH/10⁷ neutrophils. During phagocytosis, the level of GSH decreased by about 30% in 15 min. This decrease was dependent on the generation of reactive oxygen compounds, because it was not observed in the neutrophils of a patient with chronic granulomatous disease. The amount of GSSG did not increase proportionally; only about 40% of the GSH that disappeared was recovered as GSSG. From the stability of the GSH levels during phagocytosis (when large amounts of oxidative products are generated) and during incubation of neutrophils with an H₂O₂-generating system, we conclude that these cells have a large capacity to keep glutathione in the reduced form. Thus, the glutathione redox system comprises a very potent system for protection of phagocytic leukocytes against oxidative stress.

The Tripeptide γ-glutamyl-cysteinyl-glycine, or reduced glutathione (GSH), protects erythrocytes and eye lens cells against oxidative damage by its reaction with radicals and peroxides.2.3 This reaction proceeds spontaneously but is greatly accelerated by the enzyme glutathione peroxidase. The product of this reaction, the disulfide GSSG, is reduced again to GSH with NADPH in the glutathione reductase reaction. These reactions are schematically given in Fig. 1.

For several reasons, this redox system is believed to be also important in protecting phagocytic leukocytes against oxidative damage. First, during phagocytosis, these cells release large amounts of superoxide,4.5 hydrogen peroxide,6.8 and possibly other highly reactive oxygen radicals.9.14 Thus, these cells can be under heavy oxidative stress. Second, glutathione, as well as glutathione peroxidase and glutathione reductase, is present in high amounts in the cytosol of these cells.15-25 Third, deficiencies in this redox system have been found that lead to dysfunctions in the bacterialidal capacities of these cells, probably owing to oxidative damage.18.26-29 And fourth, glutathione is an important protectant of microtubule synthesis in neutrophils.23.25.30

Until now, conflicting data have been published on the amount of GSH in phagocytes and on the changes in these levels during particle ingestion.15.25.31.32 In erythrocytes, it has been shown that artifacts are easily introduced by the rapid oxidation of GSH during its isolation.33.34 Moreover, formation of mixed disulfides further complicates the study on intracellular GSH and GSSG levels.35 In this article a comparative study is described on the levels of GSH and GSSG in human neutrophils at rest and during phagocytosis, measured with three independent methods. The results indicate that only the cyclic assay with glutathione reductase specifically measures glutathione, and that the glutathione redox system effectively protects the cells against oxidative damage.

MATERIALS AND METHODS

Reduced glutathione (GSH; Boehringer, Mannheim, W. Germany) was dissolved in 1 mM EDTA. Oxidized glutathione (GSSG; Boehringer, Mannheim, W. Germany) was dissolved in 0.1 M sodium phosphate (pH 7.0). N-ethylmaleimide (NEM; Nutritional Biochemical Corp., Cleveland, Ohio) was dissolved in 0.1 M sodium phosphate buffer (pH 7.2). Serum-treated zymosan (SIZ) was prepared by incubation of zymosan with serum in phosphate-buffered saline (PBS) at a concentration of 400 μM. 5,5’-Dithiobis-(2-nitrobenzoic acid) (DTNB; Aldrich Chemical Co., Inc., Milwaukee, Wisc.) was dissolved by boiling in water. Glutathione reductase 120 U/mg (Boehringer, Mannheim, W. Germany) was diluted with 0.01 M sodium phosphate buffer (pH 7.2). Serum-treated zymosan (STZ) was prepared by incubation...
tion of zymosan (ICN Pharmaceuticals Inc., Cleveland, Ohio) with fresh normal human serum at 37°C as described before, followed by washing twice with 154 mM NaCl and suspension in 154 mM NaCl to 10 mg/ml. Formaldehyde dehydrogenase (E.C. 1.2.1.1) was isolated from beef liver as described by Koivusalo and Uotila. The fractions with formaldehyde dehydrogenase activity, but without nonspecific aldehyde dehydrogenase activity, were pooled and stored at 80°C. This preparation contained about 2 mg protein/ml with a specific activity of 0.06 U/mg protein.

**Purification of Human Neutrophils**

Neutrophils were isolated from fresh defibrinated human blood as described before. The cells were suspended in a buffer with pH 7.4, consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.6 mM CaCl₂, 1.0 mM MgCl₂, and 5.5 mM glucose. The cells were counted electronically (Coulter counter, model ZF). The final cell suspension contained more than 92% polymorphonuclear cells (PMN), the remaining cells being lymphocytes. The cell suspensions were incubated in a shaking water bath at 37°C, and the specific activity of the substrate was kept for 10 min at 37°C. Next, 0.2 ml of 10% (w/v) Na₂WO₄ in 0.1 M EDTA solution was added and mixed for 5 min. The mixture was left for 10 min at 4°C, then the sediment was spun down. The absorbance at 412 nm was measured in a mixture of 1 ml supernatant, 1.25 ml 1 M Tris-HCl (pH 8.0), and 0.1 ml 40 mg/ml DTNB. The GSH concentration in the samples was calculated by comparison with a set of GSH standards in cell medium. The variance of this determination was 1.6% (n = 10); the sensitivity was 2 μM. The recovery of GSH added to the cells just prior to lysis was 92% ± 4% (mean ± SD, n = 6).

**Measurement of the Total Amount of Glutathione**

The total amount of GSH + GSSG in neutrophils was measured with a modification of the so-called "cyclic method," earlier described by Owens and Belcher (Fig. 3). In this assay, GSH is oxidized by DTNB, and the product is reduced by NADPH in the presence of glutathione reductase.

In the original assay, the increase in absorbance at 412 nm was measured. We increased the sensitivity (from 3 to 0.02 μM) and the specificity (see Discussion) of this assay by measuring the decrease in fluorescence of NADPH (excitation 360 nm, emission >400 nm). The signal from samples of phagocytosing cells was corrected for the light scattering caused by the zymosan particles.

Samples of 0.05 ml cells (20 x 10⁶/ml) were lysed with 3 ml ice-cold 1 mM EDTA and pumped into the AutoAnalyzer system, together with the reagents described in Table 1. The GSH + GSSG concentration in the samples was calculated by comparison with a set of GSH standards in cell medium and expressed as "GSH equivalents" (1 mole GSSG = 2 mole GSH equivalents). The variance of this determination was 2.4% (n = 10). The recovery of GSH added to the cells just prior to lysis was 97% ± 5% (n = 5).

**Measurement of GSSG**

GSSG was measured in the cyclic method after removal of GSH, essentially as described by Güntherberg and Rapoport. For GSH...
GLUTATHIONE LEVELS DURING PHAGOCYTOSIS

Table 1. Composition of Reagents for the Cyclic Method

<table>
<thead>
<tr>
<th>Solution</th>
<th>Pump Speed (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M potassium-phosphate buffer + 0.1 M EDTA</td>
<td>1.58</td>
</tr>
<tr>
<td>DTNB (124 µg/ml)</td>
<td>0.22</td>
</tr>
<tr>
<td>0.01 M sodium phosphate buffer (pH 7.2)</td>
<td>0.12</td>
</tr>
<tr>
<td>glutathione reductase + 1 mM EDTA</td>
<td>0.12</td>
</tr>
<tr>
<td>Sample (cells in 1 mM EDTA)</td>
<td>0.53*</td>
</tr>
<tr>
<td>Air</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*The samples were pumped into the system at 0.8 ml/min for 40 sec, followed by water for 20 sec.

binding, N-ethylmaleimide (NEM) was added to 0.5 ml of a cell suspension (10 x 10⁶/ml) to a concentration of 130 µM. After 15 sec, a cell extract was made with 3% (w/v) perchloric acid. Thereafter, 0.5 ml of the supernatant was put on a G-10 Sephadex column (40 cm x 0.8 cm), equilibrated with 0.1 M sodium phosphate + 0.1 mM NaNO₂ (pH 7.0). The column was eluted with the same medium. The first 7 ml of the eluate were discarded; the following 9 ml, which contained the GSSG, were taken for the measurement. In this way, the GSSG in the gel filtration was 97 ± 5%. The recovery of GSSG was completely freed from the excess NEM, HClO₄, and albumin and, to a lesser extent, several other inhibiting factors of the cyclic method. The recovery of GSSG in the gel filtration was 97 ± 9% (mean ± SD, n = 11). Addition of GSH (15 µM) to the cell sample was not detectable in this assay. The sensitivity of this assay was the same as for the cyclic method.

Measurement of Hydrogen Peroxide

H₂O₂ was measured with the method described by Homan-Müller et al.

RESULTS

The amount of reduced glutathione in resting neutrophils was measured with the FDH method, the DTNB method, and the cyclic method. As shown in Table 2, the lowest values were found with the cyclic method. Clearly, other sulfhydryl compounds than GSH are measured with the first two determinations. This has been described for the DTNB method, but is also true for the FDH method: Table 3 shows that albumin and, to a lesser extent, several other compounds were detected with the DTNB and the FDH method, but not with the cyclic method. Thus, of the three assays used, the cyclic method has the highest specificity for GSH.

The amount of GSSG in resting neutrophils is very low (see Table 2). Therefore, no large error is introduced when this value is subtracted from the value for the total glutathione content. In this way, we estimate the amount of GSH in resting neutrophils to be about 13 nmole/10⁷ cells.

During incubation of resting neutrophils at 37°C, neither the total amount of glutathione nor the degree of reduction changed (Fig. 4). The amount of sulfhydral levels during phagocytosis was measured with the cyclic assay. Neutrophils (2 x 10⁷/ml) were incubated for 60 min at 37°C without (left) and with (right) serum-treated zymosan (2 mg/ml). At the times indicated, samples were taken, lysed with 1 mM EDTA, and subjected to the cyclic assay for GSH + GSSG. GSSG was isolated from parallel samples and also measured with the cyclic assay. The results are given in GSH equivalents as the mean and SD of 4 experiments. The amount of GSH was calculated as the difference between the amount of GSH + GSSG and the amount of GSSG in each experiment, and also given as the mean and SD of these 4 experiments.

Table 2. Levels of Reduced and Oxidized Glutathione in Resting Human Neutrophils

<table>
<thead>
<tr>
<th>Assay</th>
<th>GSH</th>
<th>GSH + GSSG</th>
<th>GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDH method</td>
<td>18.7 ± 5.8 (60)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DTNB method</td>
<td>15.7 ± 2.7 (12)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cyclic method</td>
<td>13.2 ± 2.8 (10)</td>
<td>14.2 ± 2.2 (21)</td>
<td>0.6 ± 0.8 (12)</td>
</tr>
</tbody>
</table>

For assays, see Materials and Methods. Values are mean ± SD of (n) experiments, in nmole/10⁷ neutrophils. The amount of GSH found with the cyclic method was calculated as the difference between the amount of GSH + GSSG and the amount of GSSG in 10 paired experiments.

GSH was also measured with two different methods in a number of identical cell samples. The results were compared in a two-sided t test for paired observations. In this way, the difference between the results of the cyclic method and the FDH method proved to be significant (p < 0.005, n = 9), as was the difference between the results of the cyclic method and the DTNB method (p < 0.002, n = 8). The results of the DTNB method and the FDH method were not significantly different from each other (p > 0.1, n = 5).

Table 3. Specificity of GSH Assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>DTNB Method</th>
<th>FDH Method</th>
<th>Cyclic Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (50 µM)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GSSG (50 µM)</td>
<td>0</td>
<td>5.9</td>
<td>206.6</td>
</tr>
<tr>
<td>Cysteine (50 µM)</td>
<td>99.7</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>Cystine (50 µM)</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>1-Ergothionine (50 µM)</td>
<td>2.8</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Ascorbic acid (50 µM)</td>
<td>0</td>
<td>7.9</td>
<td>0</td>
</tr>
<tr>
<td>Albumin (0.5%, w/v)</td>
<td>26.5</td>
<td>32.0</td>
<td>0</td>
</tr>
<tr>
<td>2-Mercapto-ethanol (50 µM)</td>
<td>105.6</td>
<td>5.7*</td>
<td>0.9</td>
</tr>
<tr>
<td>Thioglycolate (50 µM)</td>
<td>105.6</td>
<td>0*</td>
<td>1.9</td>
</tr>
</tbody>
</table>

The reaction with each compound was compared with the reaction found with GSH. The results are expressed as percentage of the reaction with GSH. The values indicated by an asterisk are taken from ref. 37.
dryl compounds measured with the FDH or the DTNB method increased, however, especially in the presence of albumin (not shown). Presumably, new thiol groups are exposed during this incubation. During phagocytosis of opsonized zymosan, the total amount of glutathione as well as the degree of reduction decreased (Fig. 4). Figure 5 shows that the decrease of GSH was dependent on the amount of zymosan added. Only about 40% of the GSH decrease was recovered as an increase in GSSG. This low recovery of GSSG was not due to the detection method: when GSH in water was oxidized by diamide, 60%–70% of the disappearing GSH was recovered as GSSG (not shown).

Neutrophils from patients with chronic granulomatous disease (CGD) ingest particles normally, but do not generate reactive oxygen compounds during this process. Neutrophils from a CGD patient. Therefore, the decrease in GSH depends on oxidative products rather than on the phagocytic process as such.

When normal neutrophils were incubated with glucose plus glucose oxidase, generating 600 nmole H₂O₂/ml/15 min, the level of GSH in the cells decreased only by 1–2 nmole/10⁷ cells; 60%–80% of the GSH that had disappeared was recovered as GSSG (Fig. 7). Nevertheless, all hydrogen peroxide generated by the glucose plus glucose oxidase was instantly degraded by the neutrophils.

DISCUSSION

The cyclic assay of GSH + GSSG measures the disappearance of NADPH fluorescence in the glut...
thione reductase reaction (see Fig. 3). Although other sulfhydryl compounds than glutathione will also react with DTNB, the reaction product R-SS-TNB cannot serve as a substrate in the glutathione reductase reaction, because only GSSG and mixed disulfides (R-SS-G) can do so. Moreover, the reduction of R-SS-TNB by GSH is also very unlikely to occur because of the competition for these sulfides by the excess DTNB. Table 3 shows that other sulfhydryl compounds than glutathione were not reactive in the cyclic assay; we have found similar results when these compounds were measured together with glutathione (not shown). Therefore, although both glutathione and other sulfhydryl compounds cause formation of 2-nitro-5-thiobenzoic acid, only glutathione leads to NADPH consumption. Thus, measurement of NADPH fluorescence not only increased the sensitivity of the assay, but also the specificity for glutathione.

The amount of GSH in resting human neutrophils has been measured before. Values of 12–18 nmole (DTNB method) and 9–19 nmole/10⁷ neutrophils (DTNB reduction in cyclic method) have been reported. We did not find as large a variation in the cells from different donors (Table 2) as others did. Values of 44–60 nmole/10⁷ neutrophils have also been reported (DTNB method); owing to nonspecific extraction procedures, other sulfhydryl compounds must have contributed to these results.

The use of defibrinated blood for the neutrophil isolation has some effect on the intracellular level of glutathione. Recently, we have found that neutrophils isolated from citrated blood have a somewhat higher glutathione content (GSH, 16.6 ± 2.0 nmole/10⁷ cells; GSSG, 0.3 ± 0.4 nmole/10⁷ cells; cyclic assay, n = 4). Possibly, the defibrination procedure to a certain extent damages the neutrophils. Our principal conclusion remains valid, however: starting with identical isolated cells, the cyclic assay for glutathione gives the most reliable results.

Reports on changes in GSH levels during phagocytosis are contradictory. Mendelson et al. described a decrease in soluble sulfhydryl compounds from 60 to 2 nmole/10⁷ human neutrophils within 15 min after zymosan addition. Our results agree with those of Burchill et al., who found a decrease of GSH in human neutrophils of about 25% in 10 min.

The drop in GSH during phagocytosis is probably related to the amount of reactive oxygen products generated by the cells. Monocytes, for instance, generate less of these products than do neutrophils, and the decrease of GSH in these cells is indeed less than in neutrophils (Voetman et al., unpublished results). Nevertheless, it is surprising that rabbit alveolar macrophages show no change in GSH levels, although these cells generate some H₂O₂.

The stability of GSH in CGD neutrophils (Fig. 6), which do not generate any reactive oxygen products at all, is in accord with the idea that these two phenomena are related. In contrast, Mendelson et al. found a decrease from 60 to 20 nmole/10⁷ CGD cells in 15 min. Apparently, the decrease during phagocytosis of sulfhydryl compounds other than GSH is caused to a large extent by nonoxidative processes.

The increase in GSSG during phagocytosis is insufficient to explain the decrease in GSH. Transport of GSSG from cells may take place but does not affect our results since our samples are taken from the total incubation mixture. As shown by Burchill et al., mixed disulfides between GSSG and RSH are formed, but we did not detect these in the cyclic assay. Nevertheless, complete recovery of GSH was not reached, even when this was taken into account. Possibly, some GSSG is further oxidized during phagocytosis. During incubation of neutrophils with a hydrogen-peroxide-generating system, a much larger percentage of the GSH disappearance was recovered as GSSG. Apparently, mixed disulfide formation and/or destruction of GSSG is less important under these conditions.

Neutrophils have a large capacity to keep glutathione in the reduced form. This conclusion is based on the observation that during phagocytosis, when large amounts of H₂O₂ are generated, as well as during incubation of neutrophils with an H₂O₂-generating system, the levels of GSH did not decrease by more than 25%-30% in 15 min. We consider this reaction to be too fast for GSH synthesis to be of any importance.

Recent studies with neutrophils from three patients who are homozygous deficient in GSSG reductase have shown a faster and more pronounced decrease of GSH in these cells during phagocytosis than in normal neutrophils. This reaction was accompanied by a short normal generation of oxygen products and by a fast inactivation of the patients’ neutrophils through oxidative damage. These results indicate that the glutathione redox system is of vital importance for the protection of neutrophils against their own oxidative, bactericidal products.

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