Endogenous Catalase Protects Human Blood Phagocytes Against Oxidative Damage by Extracellularly Generated Hydrogen Peroxide

By Alwin A. Voetman and Dirk Roos

Human blood monocytes and neutrophils were incubated with an H$_2$O$_2$-generating system (glucose plus glucose oxidase) in the presence and absence of 2 mM sodium azide, to assess the importance of catalase in the protection of these cells against heavy external oxidative stress (50 nmole H$_2$O$_2$/ml/min). Before and after these incubations, the cell integrity was determined, as well as the following cell functions: chemotactic response towards casein, and oxygen consumption and release of lysosomal enzymes during zymosan ingestion. The levels of reduced and oxidized glutathione were also measured, as were the activities of superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase.

Incubation with the H$_2$O$_2$-generating system in the absence of azide decreased the amount of reduced glutathione in the monocytes by about 25% within 30 min, with a concomitant proportional increase in oxidized glutathione, but had no appreciable effect on the extent of reduction of glutathione in the neutrophils. The cell integrity, cell functions, and enzymic activities were barely affected by this treatment in either type of cell. Incubation with the H$_2$O$_2$-generating system in the presence of azide (to inhibit endogenous catalase) resulted in a strong decrease in the level of reduced glutathione and an increase in oxidized glutathione, especially in the monocytes. The cell integrity and functional responses decreased concomitantly, but the enzymic activities remained normal. Only the glutathione reductase activity in the monocytes decreased significantly. These effects were not seen when the glucose oxidase in the H$_2$O$_2$-generating system was boiled or when azide-treated cells were washed and incubated with the H$_2$O$_2$-generating system plus catalase. Incubation with azide alone had no effect on any of the parameters tested. These results indicate that catalase is needed for adequate protection of monocytes and neutrophils against heavy external oxidative stress. Presumably, the glutathione redox cycle is mainly involved in repair of oxidized cell components, whereas catalase directly decomposes hydrogen peroxide.

During phagocytosis, monocytes and neutrophils release large amounts of hydrogen peroxide into the phagosomes and the extracellular medium. This product is used by these cells to kill a variety of microorganisms. However, because hydrogen peroxide can diffuse through the membranes of these cells, the interior of the phagocytes has to be protected against peroxidation. Two systems with potential protective capacity are known: catalase, which decomposes hydrogen peroxide into water and oxygen, and the glutathione reductase system, which converts hydrogen peroxide into water by means of reducing equivalents from NADPH.

The importance of protection of the phagocyte function against peroxidation has been described by several authors. When hydrogen peroxide, generated during phagocytosis or adherence, is scavenged with catalase, dihydroxybenzoic acid, or vitamin E, the phagocytic and chemotactic responsiveness of neutrophils is enhanced. Anaerobiosis has a similar effect. Patients with chronic granulomatous disease have neutrophils that are unable to generate hydrogen peroxide; such cells are not deactivated by high doses of chemotactic agents, which cause release of hydrogen peroxide and chemotactic deactivation in normal cells. Incubation of human neutrophils with hydrogen peroxide or with xanthine plus xanthine oxidase, a system that generates hydrogen peroxide and some superoxide, also results in functional impairment of the cells, especially when the intracellular sulfhydryls are oxidized.

So far, only little is known about the importance of catalase and the glutathione redox system in the protection of phagocytic cells. The only indication that the glutathione system might be important in this respect has been obtained from the observation that neutrophils deficient in glutathione synthetase or glutathione reductase, are damaged during phagocytosis as well as during incubation with a system that generates hydrogen peroxide. In contrast, normal cells remain functionally intact under these conditions. As to the importance of catalase as a protective cell component, it is known that inhibition of this enzyme by azide in normal neutrophils impairs neither the respiratory burst nor the release of lysosomal enzymes during phagocytosis. We have now found that neutrophils are severely damaged by an H$_2$O$_2$-generating system in the presence of azide, indicating that catalase is an important protectant of these cells against heavy oxidative stress generated extracellularly.

MATERIALS AND METHODS

Ficoll solution for monocyte purification (mol wt 70,000) was obtained from Pharmacia (Uppsala, Sweden); 350 g of it was dissolved in 800 ml of water. 150 ml of a solution of 0.175 M
OXIDATIVE DAMAGE OF HUMAN BLOOD PHAGOCYTES

Tris-HCl (pH 7.4 at 4°C) were added, and the total volume was made 1 liter with water. Two-hundred milliliters of this solution were diluted with 200 ml Eagle minimal essential medium (MEM; Gibco, Grand Island, N.Y.) fortified by 25 mM Tris (pH 7.4 at 37°C) and 1% (w/v) human albumin. The specific gravity of this solution was 1.062 g/cm³ at 4°C; the osmolality was 275 mosm.

Enzymes were obtained from Boehringer, Mannheim, West Germany. All chemicals were of analytical grade.

Cell Isolation

Neutrophils were isolated from fresh citrated human blood as described before. The cells were suspended in a buffer (pH 7.4) consisting of 138 mM NaCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.6 mM CaCl₂, 1.0 mM MgCl₂, 5.5 mM glucose and 0.5% (w/v) albumin. The final cell suspension contained more than 90% polymorphonuclear cells (PMN), the remaining cells being lymphocytes.

Monocytes were also isolated from fresh citrated human blood. By centrifugation of the leukocyte-enriched "buffy coat" over a Ficoll-Isopaque solution with a specific gravity of 1.077 g/cm³, the monocytes and lymphocytes were separated from the neutrophils and erythrocytes. The monocyte-lymphocyte fraction was washed and erythrocytes. The monocyte-lymphocyte fraction was washed with phosphate-buffered saline (PBS)-13 mM sodium citrate-1% (w/v) albumin to remove the platelets and was incubated for 30 min at 37°C in MEM-25 mM Tris-1% (w/v) albumin to remove the ingested Isopaque. The monocytes were separated from the lymphocytes by a modification (M. de Boer et al., in preparation) of the method described by Loos et al. In short, 5 x 10⁶ cells were suspended in 6 ml of an ice-cold Ficoll solution with a density of 1.062 g/cm³ at 4°C (see Materials) and put into a test tube. One milliliter of MEM-25 mM Tris was layered on this suspension, and the tube was centrifuged for 10 min at 4°C and 2200 × g. The monocytes were recovered from the interphase between the MEM-Tris and the Ficoll solution; the lymphocytes in the pellet were discarded. The monocytes were washed and resuspended in the same medium as the neutrophils. The cells were differentiated by size analysis and with light microscopy. The final cell suspension contained more than 75% monocytes and less than 2% neutrophils, the remaining cells being lymphocytes. With this method, about 70% of the monocytes were obtained.

Enzyme Determinations

Superoxide dismutase activity was measured with a modification of the method described by Salin and McComb. Cells were lysed with 0.2% (w/v) Triton X-100 and centrifuged for 4 min at 7000 g and 4°C. The particle-free supernatant was tested in various dilutions for its ability to inhibit cytochrome-c reduction by superoxide generated in the xanthine-xanthine oxidase reaction. Supernatant (400 µl) was added to 2.6 ml of a mixture of 20 mM Na₂CO₃, 0.1 mM EDTA, 3.3 mM cytochrome-c, 0.2% (w/v) Triton X-100, 50 µM xanthine, and 1 mM NaN₃. The reaction was started by addition of 10 µl of xanthine oxidase (final concentration 0.01 U/ml). The increase in absorbance at 550 nm was measured at 25°C for at least 10 min against a 100% value without cell fraction and a blank without xanthine oxidase. One unit of superoxide dismutase activity is defined as the amount of superoxide dismutase that inhibits the cytochrome-c reduction in this assay by 50%.

The activities of catalase, glutathione peroxidase, glutathione reductase, myeloperoxidase, and glucose-6-phosphate dehydrogenase (G6PD) were measured as described before.

Glutathione Assays

The total amount of reduced glutathione and oxidized glutathione (GSH plus GSSG) was measured with a modification of the so-called "cyclic method" of Owens and Belcher. In this assay, GSH is oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid) and GSSG is reduced by NADPH in the presence of glutathione reductase. In our assay, we measured the decrease in fluorescence of NADPH (excitation 360 nm, emission >400 nm). Oxidized glutathione (GSSG) was measured in the "cyclic method" after removal of GSH with N-ethylmaleimide and separation of GSSG on Sephadex G-10. Reduced glutathione (GSH) was calculated as the difference between the total amount of glutathione and the amount of oxidized glutathione.

Functional Tests

Oxygen consumption was measured with an oxygen electrode as described previously.

Cell integrity was measured with the vital stains fluorescein diacetate and ethidium bromide. Intact cells split fluorescein diacetate into fluorescein and acetate, and exclude ethidium bromide; therefore, such cells stain bright green when illuminated at 488 nm, whereas dead cells stain red owing to coupling of ethidium bromide with DNA. Fluorescein diacetate 100 µl (25 µg/ml PBS), ethidium bromide 100 µM (1 mg/ml PBS), and 100 µl of cell suspension (2 x 10⁶/ml) were mixed. After 5 min, this mixture was diluted with 1 ml of PBS. The numbers of red and green cells were detected with a cytofluorograph (Bio/Physics Systems Inc., New York, N.Y.).

Chemotaxis towards casein was measured with the leading front method as described before. The incubation times were 70 and 90 min for neutrophils and monocytes, respectively. For neutrophils, filters with a pore size of 3 µm were used (Millipore S.A., Molsheim, France; type SS); for monocytes, filters with 12 µm pore size were used (Sartorius Membranfilter GmbH, Göttingen, West Germany; type SM 125).

Release of lysosomal enzymes from phagocytosing neutrophils was measured by incubating the cells (10⁵ x 10⁶/ml) at 37°C with 1 mg serum-opsonized zymosan/ml. Samples of 0.45 ml were centrifuged in the cold, and the supernatants were stored at 4°C. Lysozyme and β-glucuronidase were measured in duplicate, as described by Goldstein et al. The cytoplasmic enzyme lactate dehydrogenase was measured as an indicator of plasma membrane integrity. Enzyme release was calculated as percentage of total enzymic activity, measured in simultaneously incubated cells to which had been added the detergent Triton X-100 (0.2%, w/v).

Cell Incubations

In all experiments, the cells (10⁷/ml) were preincubated at 37°C for 10 min in plastic tubes with 2 mM azide (to inhibit catalase) or with an equal volume of PBS (total incubation volume between 0.6 and 1.9 ml/tube). The reactions were started by addition of glucose oxidase (0.05 U/ml) to the 5.5 mM glucose-containing incubation mixtures. At 0, 30, and 60 min thereafter, the incubations were terminated by placing the tubes in ice. For each subsequent assay, a separate incubation tube was used. (Viable cells adhered more firmly to the tubes than did dead cells; therefore, the taking of several samples from one tube induced a selection of cells.) The enzymic and glutathione determinations were performed in lysates of unwashed cells to prevent the washing away of extracellular material. It was checked that the presence of glucose plus glucose oxidase and/or NaN₃ had no appreciable effect on the enzymic and glutathione assays, except for catalase, which was inhibited for 80%–90% by the azide, and glucose-6-phosphate dehydrogenase (G6PD), which was inhibited by glucose plus glucose oxidase (the H₂O₂ formed, oxidized the NADPH generated by G6PD). The cell integrity, oxygen consumption, release of lysosomal enzymes, and chemotaxis were tested with cells that had been washed twice.
Table 1. Enzymic Activities in Neutrophils and Monocytes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Neutrophils</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase*</td>
<td>208-493</td>
<td>118-195</td>
</tr>
<tr>
<td>GSH peroxidase†</td>
<td>8-15</td>
<td>6-8</td>
</tr>
<tr>
<td>GSSG reductase†</td>
<td>14-37</td>
<td>7-19</td>
</tr>
<tr>
<td>G6PD†</td>
<td>97-200</td>
<td>30-43</td>
</tr>
<tr>
<td>Myeloperoxidase*</td>
<td>10-17</td>
<td>4-7</td>
</tr>
<tr>
<td>Superoxide dismutase‡</td>
<td>5-26</td>
<td>11-31</td>
</tr>
</tbody>
</table>

n, Number of experiments with cells from different donors.

*In U/10⁷ cells at 25°C.
†In IU/10⁷ cells at 25°C.
‡In U/10⁷ cells at 25°C, as defined in Materials and Methods.

Enzymic Activities

To evaluate the cause and significance of these changes in the extent of reduction of glutathione, some enzymic activities and functional parameters of the cells were measured. Table 1 shows the activities of several enzymes involved in the handling of hydrogen peroxide and of superoxide. The activity of most enzymes, especially of glucose-6-phosphate dehydrogenase, was higher in the neutrophils than in the monocytes.

During the incubations with glucose plus glucose oxidase with or without azide, the activities of glutathione peroxidase and superoxide dismutase did not change (not shown). Catalase activity did not change either, but could only be tested in the incubations without azide (see Materials and Methods). Incubation with azide alone had no effect on the other enzyme activities (not shown). The activity of glutathione reductase in the neutrophils was not affected by the oxidative stress; in the monocytes, it decreased to 45% of the original activity when glucose oxidase and azide were both added (Fig. 2). Glucose-6-phosphate dehydrogenase activity could not be measured in the presence of glucose plus glucose oxidase (see Materials and Methods).

Functional Assays

As Fig. 3 shows, the cells remained intact when incubated with glucose plus glucose oxidase. When azide was also added, the neutrophils lost the ability to exclude ethidium bromide, but monocytes were much less affected in this respect. However, neutrophils and monocytes that did exclude ethidium bromide after incubation with glucose plus glucose oxidase plus azide, to a great extent lost the ability to hydrolyze fluorescein diacetate (not shown). Incubation with azide alone had no effect (not shown).

To check the specificity of H₂O₂ as the damaging agent, the last experiment was repeated with cells incubated with boiled glucose oxidase plus azide and...
Fig. 2. Activity of glutathione reductase. The GSSG reductase activity is given as percentage of the activity in cells that had not been incubated (neutrophils, 19.9 ± 5.0 lU/10⁶ cells at 25°C, mean ± SD, n = 4). The GSSG reductase activity was measured directly in the lysates of the unwashed cells. (○) Cells incubated with PBS plus glucose (5.5 mM). (●) Cells incubated with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM). (▲) Cells incubated with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM) plus NaN₃ (2 mM). Results are the means of 4 paired experiments ± SEM. For statistical significance, see legend to Fig. 1.

Fig. 3. Exclusion of ethidium bromide. The cell integrity is given as percentage of the values obtained with cells that had not been incubated (neutrophils and monocytes, 99% ± 1% exclusion of ethidium bromide, mean ± SD, n = 4). The number of red cells was measured and corrected for the number of cells left after the incubation. The cells were washed twice before being tested. (○) Cells incubated with PBS plus glucose (5.5 mM). (●) Cells incubated with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM). (▲) Cells incubated with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM) plus NaN₃ (2 mM). Results are the means of 4 paired experiments ± SEM. For statistical significance, see legend to Fig. 1.

Fig. 4. Involvement of H₂O₂ in the process of cell damage. The cell integrity was measured by exclusion of ethidium bromide. For experimental details, see legend to Fig. 3. Before being tested, the cells were incubated at 37°C, as follows: (A) with PBS plus glucose (5.5 mM) for 0 and 60 min; (B) with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM) plus azide (2 mM) for 60 min; (C) with glucose oxidase (0.05 U/ml, heated for 15 min at 100°C) plus glucose (5.5 mM) plus azide (2 mM) for 60 min; (D) with azide (2 mM) for 10 min, washed once, then with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM) for 60 min; (E) with azide (2 mM) for 10 min, washed once, then with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM) plus catalase (3000 U/ml) for 60 min. All results were obtained in one experiment.

Fig. 5. Chemotactic activity towards casein. Results given in percentage of the values obtained with cells that had not been pretreated (neutrophils, 63 ± 18; monocytes, 36 ± 8 μm; mean ± SD, n = 4). Spontaneous mobility without casein was subtracted from the mobility observed in the presence of casein. The cells were washed twice before being tested. (○) Cells incubated with PBS plus glucose (5.5 mM). (●) Cells incubated with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM). (▲) Cells incubated with glucose oxidase (0.05 U/ml) plus glucose (5.5 mM) plus NaN₃ (2 mM). Results are the means of 4 paired experiments ± SEM. For statistical significance, see legend to Fig. 1.
Figure 6 shows the capacity of the cells to increase their oxygen consumption during an incubation with phagocytosable particles. Incubation with only glucose plus glucose oxidase decreased this capacity in neutrophils, but not in monocytes. When azide was also present, both types of cell were totally inactivated within 30 min. Similar results were obtained when phorbol myristate acetate was used as the respiratory-stimulating agent (not shown). Incubation with azide alone had no effect (not shown).

The release of lysosomal enzymes by neutrophils during phagocytosis of serum-opsonized zymosan was also tested. Pretreatment of the cells with glucose plus glucose oxidase, either with or without azide, decreased the capacity of the cells to release lysosomal enzymes, especially β-glucuronidase (Table 2). Simultaneously, the leakage of lactate dehydrogenase from the cells increased, confirming the results obtained with ethidium bromide. Incubation with azide alone had no effect (not shown).

**DISCUSSION**

The results of this study show that human blood phagocytes are damaged by extracellularly generated hydrogen peroxide (50 nmole/ml/min) when the catalase in the cells is inhibited. This rate of hydrogen peroxide production is comparable to that observed with 10^7 human neutrophils/ml during phagocytosis. Therefore, this oxidative stress is not excessive, although its duration was longer than that of the respiratory burst of phagocytosing cells.

The fact that we measured cell damage under the conditions employed in this study, but not with phagocytosing cells (in the absence or presence of azide), may thus be due either to the duration of the oxidative stress or to the extracellular site of H_2O_2 production.

From the results of our study it appears that the plasma membrane is a vulnerable part of the phagocytes. The cell integrity, the chemotactic responsiveness, as well as the oxygen-consuming system of these cells were damaged by the incubations with H_2O_2 plus azide, and each of these functions is connected in some way to the plasma membrane. Even the glutathione reductase, which was rapidly inactivated in monocytes, may be associated with the cell membrane, as it is, to some extent, in erythrocytes (G.E.J. Staal, personal communication).

The chemical nature of the membrane damage is not known. We showed that H_2O_2 is essential to this process, since boiling of glucose oxidase or addition of catalase prevented the damage. Moreover, the fact that the damage took place in the presence of azide implies that myeloperoxidase-mediated reactions are not involved, since azide inhibits this enzyme. Therefore, lipid peroxidation by H_2O_2 itself is a likely possibility. Whether superoxide (O_2^-) may also be harmful to phagocytes is still open to discussion.

A role for myeloperoxidase in the protection against oxidative injury was excluded by recent experiments with catalase-deficient neutrophils: although these cells contained a normal amount of myeloperoxidase, they were much more sensitive to external oxidative stress than control cells.
Our results prove that endogenous catalase protects neutrophils and monocytes against peroxidation from an extracellular source. During phagocytosis, when these cells generate H₂O₂ themselves, azide has no effect; thus, catalase does not seem essential for the preservation of the cell functions under these conditions. The same conclusion was reached from our experiments with catalase-deficient neutrophils (D. Roos et al., submitted). Probably, the glutathione redox system can adequately protect the phagocytes during particle ingestion, but a certain level of H₂O₂ may build up in the cells during extracellular H₂O₂ generation. In that case, catalase will be essential for cell protection. Enzymatically, this also makes sense, since catalase cannot be saturated by its substrate: to a certain limit, this enzyme works faster when more H₂O₂ is available.

When the glutathione redox system is blocked, either by glutathione reductase deficiency or by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (R. van Zwieten et al., unpublished observations), the H₂O₂ release by phagocytosing neutrophils is not abnormally high. This indicates that the glutathione system is not reacting directly with H₂O₂ itself. Possibly, in phagocytosing cells, this system is involved mainly in reducing oxidized cell components. Only during heavy external oxidative stress is catalase needed for extra protection.

In conclusion, the relative importance of catalase and the glutathione redox system in the protection of phagocytes against oxidative damage can be summarized as follows. Normal cells are not harmed by H₂O₂ generated either by the phagocytes or in the medium. Cells with a genetic abnormality in the glutathione redox system are damaged by H₂O₂ generated either intra- or extracellularly. Catalase-deficient or catalase-inhibited cells are damaged only by an extracellular source of H₂O₂ (this report; Roos et al.28)

ACKNOWLEDGMENT

We thank Drs. H. K. Prins, J. A. Loos, R. S. Weening, and M. N. Hamers for valuable advice.

REFERENCES

25. Goldstein IM, Roos D, Kaplan HB, Weissmann G: Complement and immunoglobulins stimulate superoxide production by...
26. Burchill BR, Oliver JM, Pearson CB, Leinbach ED, Berlin RD: Microtubule dynamics and glutathione metabolism in phagocy-
447, 1978
27. Jandl RC, André-Schwartz J, Borges-DuBois L, Kipnes RS, 
Endogenous catalase protects human blood phagocytes against oxidative damage by extracellularly generated hydrogen peroxide

AA Voetman and D Roos