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Polymorphonuclear Leukocyte Bactericidal Activity and Oxidative Metabolism During Glutathione Peroxidase Deficiency

D. A. BASS,[1]* L. R. DECHATELET,[1] R. F. BURK,[2] P. SHIRLEY,[1] AND P. SZEJDA[1]

Departments of Medicine and Biochemistry, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103,[1] and Liver Unit, Department of Medicine, University of Texas Southwestern Medical School and Veterans Administration Hospital, Dallas, Texas 75235[2]

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Glutathione peroxidase (GPx) deficiency has been proposed as a cause of some instances of chronic granulomatous disease (CGD). GPx activity varies greatly among species, and specific deficiency of this selenium-dependent enzyme can be produced by dietary selenium deficiency in rats. Bactericidal activity of polymorphonuclear (PMN) leukocytes from normal rats, humans, and guinea pigs (GPx high, intermediate, and nearly absent, respectively), selenium-deficient rats (GPx absent), and a patient with CGD were compared. There was no correlation between natural levels of GPx and bactericidal activity; only CGD was associated with inability to kill a *Proteus mirabilis* strain in vitro (killing known to be dependent on oxidative mechanisms). Postphagocytic metabolism was examined in normal and GPx-deficient rats. Both demonstrated normal iodination and superoxide production during phagocytosis and gave similar histochemical reduction of nitroblue tetrazolium dye under either resting or endotoxin-stimulation conditions. Postphagocytic hexose monophosphate shunt activity was somewhat lower in PMN from GPx-deficient animals as compared with normal but was substantially (10-fold) higher than that observed in resting cells. Thus, postphagocytic oxidative responses and subsequent bactericidal activity of PMN leukocytes were not compromised by complete absence of GPx, even in the species with the highest natural level of this enzyme. These results are not compatible with the hypothesis that CGD can be caused by a deficiency of GPx.

Polymorphonuclear neutrophil leukocyte (PMN) phagocytosis and killing of microbes is normally accompanied by a burst of oxidative metabolism. This metabolic burst is manifested by increased oxygen consumption (17) and production of oxidative substances such as hydrogen peroxide (15) and superoxide (1), by the concomitant increase in fixation of iodide to precipitable proteins (iodination) (18), and the reduction of nitroblue tetrazolium (NBT) dye (4). These events are accompanied by a marked stimulation of the hexose monophosphate shunt (HMPS) (17). Inability to produce such a metabolic burst is associated with a significant defect in microbial killing and is characteristic of chronic granulomatous disease (CGD) (13, 28). A deficiency of any of several enzymes may interrupt this oxidative PMN response and result in a similar syndrome. Enzyme deficiencies that have been suggested include a reduced pyridine nucleotide oxidase (3, 26), glucose-6-phosphate dehydrogenase (6), and glutathione peroxidase (GPx) (14, 22).

GPx is present within the cell cytosol (14, 29), where it achieves the reduction of hydrogen peroxide or lipid hydroperoxide with concomitant oxidation of glutathione. Although the roles of GPx are currently being clarified, it is usually visualized as an antioxidant, protecting the cell by destruction of potentially toxic peroxides (16, 23). However, GPx has also been proposed as one of the enzymes in the metabolic cycle leading to production of oxidative substances and resulting in bacterial killing by PMN (14, 22, 34). A fungicidal defect of GPx-deficient rat leukocytes was suggested by the preliminary studies of Serfass and Ganther (32). They employed a histochemical method for examining intracellular survival of candida organisms; however, the data suggested only very poor killing (2 to 20%) in the normal rat phagocytes and a mild impairment of killing by the deficient cells; oxidative metabolic responses were not examined.

GPx levels vary widely among species. Rats demonstrate a high natural level of the enzyme; humans have an intermediate level, and in

guinea pigs the enzyme is nearly absent (2, 11, 31). Furthermore, leukocyte GPx is selenium dependent, and nearly complete GPx deficiency may be induced in rodents by feeding a selenium-deficient diet (12). The current studies employed normal rat, guinea pig, and human neutrophils, neutrophils from GPx-deficient rats, and neutrophils from a patient with chronic granulomatous disease, in an attempt to evaluate the role of GPx in the bactericidal activity of the cell.

MATERIALS AND METHODS

Experimental animals. Weanling male Holtzman rats were fed ad libitum a *Torula* yeast-based selenium-deficient diet (5), or the same diet supplemented with 0.5 mg of selenium as sodium selenite per kg as a "diet control," and provided tap water. These diets were fed for 6 months before initiation of the experimental studies. Lab chow control rats were fed Purina lab chow. The diets were well tolerated. Although animals on both artificial diets weighed slightly less than those fed lab chow, the animals on either selenium-adequate or selenium-deficient diets were healthy, appeared identical, and were free of infection. Guinea pigs were obtained from Perfection Breeders (Douglasville, Pa.), and fed Purina guinea pig feed.

Isolation of leukocytes. Rat and guinea pig neutrophil leukocytes were obtained from peritoneal exudates induced by two injections of 5 ml of 1% peptone (rats) or 15 ml of 0.5% glycogen (guinea pigs) 18 and 3 h before sacrifice. The animals were sacrificed by intracardiac injection of air under light ether anesthesia. Peritoneal cavities were opened and lavaged with calcium- and magnesium-free Hanks balanced salt solution containing 1% gelatin (CaMg-free HBSSg) and heparin, 20 U/ml. Peritoneal exudates were pooled and washed twice with CaMg-free HBSSg with centrifugation at 400 × *g* for 10 min. The cells were suspended in HBSSg at a concentration of 10^7 PMN per ml.

Normal human leukocytes were isolated from 30 ml of heparinized blood obtained from normal donors. Leukocyte separation was achieved by layering 10 ml of whole blood over 10 ml of 9.6% sodium metrizoate and 5.6% Ficoll (Lymphoprep, Nygaard, Oslo, Norway). Without centrifugation, the erythrocytes agglutinated at the metrizoate-blood interface and fell into the lower layer; after 20 to 40 min, the leukocyte-rich plasma was removed, washed twice with CaMg-free HBSSg, and suspended in HBSSg at a concentration of 10^7 PMN/ml. Leukocytes were similarly obtained from a patient (S.S.) with documented chronic granulomatous disease and normal GPx levels whose condition has been reported previously (10).

Bactericidal assay. A modification of the method of Maaløe (21) was employed. The test organism was a strain of *Proteus mirabilis* (kindly provided by Jim Smith, Dallas, Tex.) that had previously been demonstrated to be excellent for assay of phagocytosis by rat leukocytes (33). Bacteria were grown in Penassay broth (Difco, Detroit, Mich.) for 18 h, washed, and suspended in 0.1% gelatin to a concentration that produced an optical density (OD) of 0.60 at 620 nm and that contained 5×10^9 to 10×10^9 colony-forming units/ml. This was diluted 1:100 before use. Fresh homologous serum was used in each assay at 10% concentration. Bactericidal assays were conducted in triplicate in plastic tubes (17 by 100 mm; Falcon no. 2025, Falcon Plastics, Oxnard, Calif.), which contained 0.1 ml of leukocytes (containing 10^6 PMN), 0.1 ml of bacterial suspension (containing 5×10^6 to 10×10^6 colony-forming units), 0.1 ml of homologous serum, and 0.7 ml of HBSSg. Control tubes contained the same concentration of serum and bacteria but lacked cells. The mixtures were incubated with continuous gentle rotation at 37°C. For determination of viable bacteria, cells were lysed by addition of 9 ml of 0.1% gelatin; after holding at room temperature for 5 min, 1 ml of this suspension was further diluted and agar pour plates were prepared. Agar overlays were prepared to prevent swarming of the *Proteus* strain.

The ability of the leukocytes to reduce NBT dye was examined under resting and endotoxin stimulation conditions by the histochemical method of Park et al. (25).

The oxidation of [1-^{14}C]glucose to $^{14}CO_2$ was determined in resting cells and cells exposed to opsonized zymosan, as previously described (9). Results are expressed as counts per minute of $^{14}CO_2$/h per 5×10^6 PMN.

Iodination was determined by the ability of cells to incorporate ^{125}I into trichloroacetic acid-precipitable material under either resting or phagocytizing conditions (27). Results are expressed as counts per minute of ^{125}I incorporated per h per 5×10^6 PMN.

The generation of superoxide anion was determined by means of the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* as described by Babior et al. (1). Each incubation flask contained 100 nmol of cytochrome *c* in a total volume of 3.0 ml of Dulbecco phosphate-buffered saline (PBS). Incubation was for 30 min at 37°C; experiments employed 5×10^6 PMN or 10^6 PMN as described in Table 6. Since virtually no superoxide production was detected by resting cells in our system, data are presented only for cells challenged with opsonized zymosan. All values are expressed as the ΔOD_{550} per 30 min where ΔOD equals the absorbance in the absence of SOD minus the absorbance in the presence of 50 μg of SOD.

GPx levels were determined on sonicated leukocytes by a modification of the method of Paglia and Valentine (24) employing H_2O_2 as substrate and the method of Lawrence and Burk (19) employing cumene hydroperoxide. Protein content of the samples was determined by the method of Lowry et al. (20). Each sample was assayed at three different levels of protein and the results were averaged. Results are given as nanomoles of reduced nicotinamide adenine dinucleotide phospahte (NADPH) oxidized per minute per milligram of protein. All assays were performed in triplicate.

RESULTS

GPx levels of normal human, guinea pig, rat, and selenium-deficient rat neutrophil leukocytes. GPx levels determined on the rat

leukocytes used in these studies are shown in Table 1. These results are in agreement with previously reported studies of these species and demonstrate a high level of GPx in normal rats, and essentially complete absence of this enzyme in selenium-deficient rats, using either H_2O_2 or cumene hydroperoxide as substrate. Normal human leukocytes contained an intermediate activity of GPx (73 nmol/min per mg) (Table 2). Guinea pig leukocytes demonstrated a barely detectable level of GPx (Table 2); in these preparations, some activity did appear to be present (27 nmol/min per mg of protein), which was linear with protein concentration.

Bactericidal activities. The ability of each leukocyte preparation to kill *P. mirabilis* is demonstrated in Fig. 1 to 3. Normal human leukocytes appear to be somewhat more efficient than normal guinea pig and rat leukocytes in this assay system (Fig. 1); however, there was no correlation between bactericidal ability and the level of glutathione peroxidase as both rat (highest GPx level) and guinea pig (lowest GPx) leukocytes demonstrated similar killing abilities.

Induction of GPx deficiency in a species with a high natural level of the enzyme was accomplished by feeding rats a selenium-deficient diet for 6 months. Peritoneal leukocytes from these GPx-deficient animals, PMNs from rats fed the same diet with selenium supplementation, and PMNs from rats fed laboratory chow were examined for bactericidal activity (Fig. 2). Pools of peritoneal cells from each of these groups were studied on four separate occasions. On each occasion, all experimental groups showed 90 to 95% killing of the test organism after 2 h. In all experiments, the GPx-deficient cells killed at least as well as the control cells.

To ascertain that oxidative metabolic responses were required for killing the strain used in these studies, bactericidal activity of cells obtained from a patient with documented chronic granulomatous disease was examined. Killing of the test organism by these cells was severely impaired (Fig. 3).

HMPS stimulation. The measurement of activity of the HMPS pathway of rat leukocytes was determined by the metabolism of [1-^{14}C]-glucose. Preliminary studies examined metabolic responses to ingestion of particles of latex, heat-killed bacteria, or opsonized zymosan (Table 3). These demonstrated very minimal HMPS stimulation after phagocytosis of latex particles but a significant response to phagocytosis of heat-killed *Proteus* organisms and the greatest response after ingestion of opsonized zymosan. Opsonized zymosan was therefore chosen for the test particles. Stimulation of the HMPS in normal and GPx-deficient rats was examined in three separate experiments that employed pooled leukocytes from a minimum of three animals in each group and triplicate determinations within each experiment (Table 4). In all instances, there was a dramatic (greater than 10-fold) increase in HMPS activity by control cells after ingestion of opsonized zymosan. Cells from rats fed the synthetic diets (with or without Se) demonstrated somewhat lower levels of HMPS stimulation, the GPx-deficient animals showing the lowest stimulation observed. Nevertheless, all animals studied showed approximately a 10-fold stimulation when comparing resting and phagocytizing leukocytes.

Iodination. Incorporation of ^{131}I into trichloroacetic acid-precipitable protein provides an indication of oxidative activities and may be directly involved in bactericidal mechanisms by the myeloperoxidase-hydrogen peroxide-halide system (18). The measurement of such incorporation of iodine in normal or GPx-deficient rat leukocytes is shown in Table 5. In all experiments, there was a large increase in incorporation of iodine into protein after phagocytosis of opsonized zymosan, and there was no significant difference between GPx-deficient, diet control, and lab chow-fed animals.

TABLE 1. *GPx levels of rat PMN leukocytes*

Source	nmol of NADPH oxidized per min per mg of protein[a]					
	Expt 1		Expt 2		Expt 3	
	H_2O_2	Cumene-OOH	H_2O_2	Cumene-OOH	H_2O_2	Cumene-OOH
Lab chow control						
1	351	190	290	186	340	259
2	351	232	279	225	253	194
Se+ diet control	394	257	372	242	484	361
Se−deficient diet	4	3	0	6	24	22

[a] Each value is the mean of triplicate determinations run at different levels of protein on a pool of cells obtained from three animals. Each study was run on three separate occasions; experiments 1, 2, and 3 refer to each of these. The conditions employed in each were the same. Cumene-OOH, Cumene hydroperoxide.

TABLE 2. *GPx levels of normal human, guinea pig, and rat PMN leukocytes*

Species	nmol of NADPH oxidized per min per mg of protein with assay substrate:	
	H_2O_2	Cumene-OOH
Rat	300[a]	222
Human	73	70
Guinea Pig	27	0

[a] Each value is the mean of triplicate determinations run at different levels of protein.

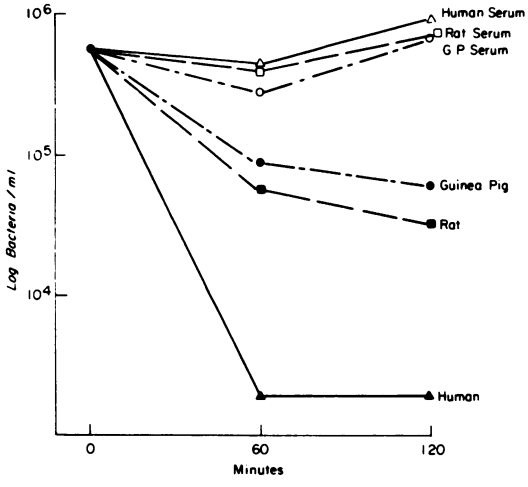

FIG. 1. *Bactericidal activity against Proteus mirabilis of normal human blood PMNs, normal guinea pig peritoneal PMNs, and normal rat peritoneal PMNs. GPx levels were 73, 27, and 276 nmol/min per mg, respectively. Homologous serum was employed in each assay for opsonin. Each point is the mean of triplicate determinations.*

Superoxide production. Superoxide production was assayed by the SOD-sensitive reduction of cytochrome *c* after phagocytosis of opsonized zymosan. Normal (lab chow control), semisynthetic diet control, and GPx-deficient rat leukocytes all generated substantial quantities of superoxide (Table 6). Observed values were on the order of magnitude previously reported for superoxide production by phagocytosing human, rabbit, and guinea pig neutrophil leukocytes (8). Leukocytes obtained from rats fed semisynthetic diets produced similar amounts of superoxide, whether GPx was normal or absent.

Reduction of NBT dye. Ability of the GPx-deficient and diet control leukocytes to reduce NBT dye under resting and endotoxin stimulation conditions was examined histochemically (Table 7). As expected, there was a very little NBT reduction in resting cells and a considerable increase after endotoxin stimulation. The

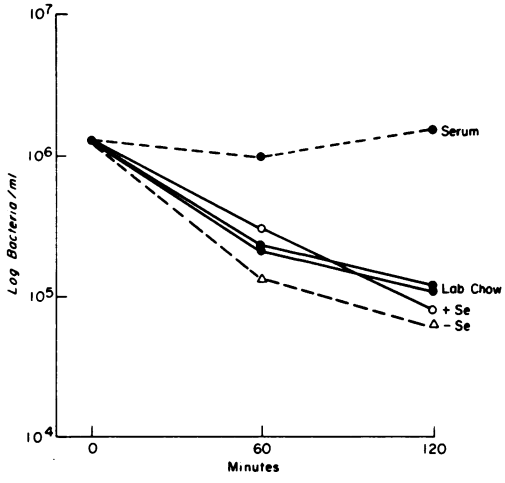

FIG. 2. *Bactericidal activity of normal (lab chow control) rat peritoneal PMNs, PMNs obtained from rats on the selenium-adequate diet and GPx-deficient PMNs obtained from rats fed a selenium-deficient diet. Each point is the mean of four experiments, each employing triplicate determinations on pools of cells from three animals in each group.*

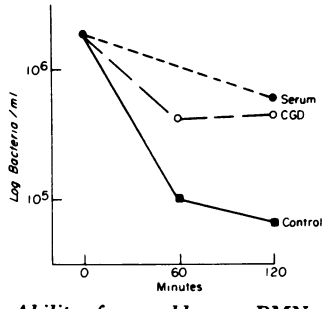

FIG. 3. *Ability of normal human PMNs and PMNs obtained from a patient with CGD to kill the test organism. Each point is the mean of triplicate determinations.*

TABLE 3. *Effect of particle type on stimulation of [1-^{14}C]glucose oxidation by rat leukocytes*[a]

Description	cpm in $^{14}CO_2$	
	Expt 1	Expt 2
Resting cells	2,081	1,751
Phagocytosing		
Latex	2,577	1,911
Proteus	14,826	8,841
Opsonized zymosan	23,248	18,883

[a] All particles were suspended in PBS to an OD_{525} of 1.0. Each assay employed 1.0 ml of these suspensions in a total volume of 3 ml containing 5×10^6 PMN/ml. Different normal rats were used in each experiment. All values represent means of closely agreeing triplicate determinations.

TABLE 4. *Oxidation of [1-^{14}C]glucose by normal and GPx-deficient rat neutrophils*

Description	cpm in $^{14}CO_2$					
	Expt 1[a]		Expt 2		Expt 3	
	Rest[b]	Phago[b]	Rest	Phago	Rest	Phago
Lab chow control						
1	1,886	22,176	1,521	32,223	1,959	37,445
2	2,379	26,540	1,569	30,931	1,894	32,892
Se+ diet control	1,504	17,642	1,224	25,538	1,168	27,203
Se−deficient	1,898	14,073	1,196	14,506	2,058	25,603

[a] Each value represents the mean of triplicate determinations. Experiments 1, 2, and 3 refer to the same groups of animals as cited in Table 1.
[b] Rest, Resting leukocytes; Phago, phagocytosing leukocytes.

TABLE 5. *Incorporation of ^{125}I into trichloroacetic acid-insoluble material*

Description	Iodination (cpm)					
	Expt 1[a]		Expt 2		Expt 3	
	Rest[b]	Phago	Rest	Phago	Rest	Phago
Control 1	1,570	39,173	3,064	57,322	800	53,810
Control 2	1,520	61,157	3,461	56,316	891	43,098
Se+ diet control	1,732	47,247	3,306	41,401	ND[c]	ND
Se−deficient	1,750	35,933	3,395	51,173	836	34,744

[a] Each value represents the mean of closely agreeing triplicate determinations. Experiments 1, 2, and 3 refer to the same groups of animals cited in Table 1.
[b] Rest, Resting cells; Phago, phagocytosing cells.
[c] ND, Not determined.

TABLE 6. *Generation of superoxide by normal and GPx-deficient rat leukocytes*

Cell source	Cytochrome *c* reduced (ΔOD/30 min)[a]	
	Expt 2	Expt 3
Lab chow control		
1	0.232	0.131
2	0.265	0.145
Se+ diet control	0.174	0.096
Se−deficient	0.138	0.133

[a] ΔOD, Difference in absorbance at 550 nm after incubation in presence and absence of SOD. Values are for phagocytosing cells; very little O_2^- was produced by resting cells. Each value represents the mean of duplicate determinations. Experiments 2 and 3 refer to the same groups of animals cited in Table 1. Experiment 2 employed 5×10^6 PMN per incubation. Experiment 3 employed 10^6 PMN/incubation.

GPx-deficient and control animals demonstrated comparable degrees of NBT reduction in both conditions.

DISCUSSION

These studies demonstrate that the rat leukocytes are capable of considerably more potent metabolic responses than previously reported. Reed and Tepperman (30) noted that ingestion of latex particles by rat leukocytes induced only a transient and mild increase in HMPS activity. Such a submaximal metabolic response has been observed after ingestion of unopsonized particles by guinea pig peritoneal PMN, rabbit peritoneal PMN, and human peripheral blood PMN (8). The present studies demonstrate that ingestion of opsonized zymosan particles by rat peritoneal PMN is followed by a substantial stimulation of [1-^{14}C]glucose metabolism, superoxide production, protein iodination, and NBT reduction. Thus, the rat peritoneal PMN leukocyte is a satisfactory cell for study of postphagocytic met-

TABLE 7. *Reduction of NBT dye by normal and GPx-deficient rat neutrophils*[a]

Leukocyte source	% Positive cells	
	Resting	Endotoxin-stimulated
Se+ diet control		
1	3	14
2	1	17
3	1	25
Se−deficient		
1	1	12
2	3	14
3	1	14

[a] This assay was run on three individual diet control and three selenium-deficient animals.

abolic changes. The rat has a high natural level of GPx; furthermore, nearly complete GPx deficiency can be induced by feeding a selenium-deficient diet. Thus, this provides an excellent experimental model for studying any alteration in postphagocytic metabolic responses induced by the removal of GPx.

The sequence of metabolic events that follow phagocytosis is controversial. Two fundamentally different schemes have been proposed. The first views that the initial event after phagocytosis is the formation of active oxidative products, e.g., hydrogen peroxide or superoxide, by the oxidation of a reduced pyridine nucleotide by an oxidase enzyme (3, 26). Excess hydrogen peroxide might diffuse from the phagosome into the cytosol and react with glutathione in the presence of GPx to form oxidized glutathione and water, thus destroying potentially toxic, excess hydrogen peroxide. Reduction of the oxidized glutathione would be accompanied by production of NADP, which in turn would stimulate the HMPS (16, 29). By this scheme the glutathione system would provide a homeostatic mechanism to remove excess oxidative substances from the cell cytosol.

Strauss et al. (34) suggested an alternative hypothesis. They proposed that the initial event after phagocytosis was an increase of glutathione reductase activity. This would allow the oxidized glutathione (GSSG) and NADPH present to form increased quantities of NADP, which would stimulate the HMPS and provide increased NADPH, perhaps in a new location or in a somehow differently active form. The new NADPH would then react with NADPH oxidase to produce hydrogen peroxide. A proportion of hydrogen peroxide so produced would be seen to react with reduced glutathione in the presence of GPx to form the GSSG necessary at the first step of the reaction. The scheme was accepted by Holmes et al. (14), who viewed it as a means of explaining their observation of decreased GPx activity in the leukocytes of female patients with chronic granulomatous disease. They viewed the removal of GPx as producing a break in the normal cycle necessary for the regeneration of GSSG.

The present studies strongly suggest that the glutathione system is not required for the initiation of oxidative responses involved in bactericidal activity. Ability to kill a *P. mirabilis* strain did not correlate with widely variable natural levels of GPx of rat, human, or guinea pig leukocytes, whereas a patient with chronic granulomatous disease and known normal GPx level demonstrated nearly absent bactericidal ability against the same strain. Moreover, whereas normal rat leukocytes contain a high level of GPx, induction of GPx deficiency did not significantly impair the ability of the cells to kill the test organisms and did not reduce postphagocytic oxidative responses (iodination, superoxide production, and NBT reduction). Recently, McCallister et al. reported that GPx-deficient rat leukocytes had postphagocytic stimulation of HMPS and NBT reduction which was similar to that observed in controls (J. McCallister, L. A. Boxer, and R. L. Braehner, Clin. Res. 24:381A, 1977).

The two metabolic schemes described above also suggest differing roles for the postphagocytic stimulation of the HMPS. The scheme of Strauss et al. (34) would suggest the HMPS stimulation occurs early in the chain of postphagocytic metabolic events and has a causal role in the formation of hydrogen peroxide. However, previous studies demonstrated that inhibition of the HMPS with colchicine (probably by blocking glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) did not alter leukocyte bactericidal activity against *Staphylococcus albus, Escherichia coli,* or *Serratia marcescens* (7). In the present studies, HMPS activity was substantially (10-fold) higher in phagocytosing GPx-deficient leukocytes than observed in resting cells. Nevertheless, this increase was slightly lower than that observed in rat leukocytes with normal GPx levels. This is compatible with the concept that a portion of the stimulation of the HMPS is normally dependent upon production of NADP during removal of excess H_2O_2 by the glutathione system (14, 16, 29). The fact that there was a 10-fold increase in HMPS activity after phagocytosis suggests that this HMPS stimulation is not entirely dependent on the glutathione system mechanism. It may be that part of the HMPS stimulation follows production of NADP by the oxidase responsible for the initial step in the production of H_2O_2 by the phagocytosing leukocyte.

The factors that regulate normal levels of GPx in PMN leukocytes are largely unknown. Low levels of GPx have been observed in some patients whose leukocytes fail to produce H_2O_2 (14, 22); however, a recent examination of seven patients with CGD revealed normal GPx levels in each (10). If the major role of leukocyte GPx is the removal of excess H_2O_2, it may be that the low GPx levels observed in some patients with CGD were secondary to the absence of normal H_2O_2 production. Thus, the low GPx in some cases of CGD might reflect the fact that GPx is not required in a cell that is incapable of normal oxidative responses. The cause of the metabolic defect responsible for these cases of CGD must be sought elsewhere.

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