NADPH Oxidase Deficiency in X-Linked Chronic Granulomatous Disease

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ABSTRACT We measured the cyanide-insensitive pyridine nucleotide oxidase activity of fractionated resting and phagocytic neutrophils from 11 normal donors, 1 patient with hereditary deficiency of myeloperoxidase, and 7 patients with X-linked chronic granulomatous disease (CGD). When measured under optimal conditions (at pH 5.5 and in the presence of 0.5 mM Mn++,), NADPH oxidase activity increased four-fold with phagocytosis and was six-fold higher than with NADH. Phagocytic neutrophils from patients with CGD were markedly deficient in NADPH oxidase activity.

INTRODUCTION

X-linked chronic granulomatous disease (CGD),1 a hereditary disorder of male children, is characterized by serious susceptibility to infection (2). Although neutrophils (PMN) from patients with CGD ingest microorganisms effectively, they fail to kill many bacteria and fungi at a normal rate (3, 4). Phagocytosis by normal PMN is associated with major changes in oxidative metabolism, including increased cyanide-insensitive consumption of oxygen (5–7), stimulation of glucose oxidation via the hexose monophosphate shunt (HMPS) (7, 8), increased generation of hydrogen peroxide and superoxide (9–11), and augmented reduction of the redox dye, nitroblue tetrazolium (NBT) (12). All of these features of increased oxidative metabolism are deficient in CGD PMN (12–13).

The primary enzyme of the phagocytic oxidative burst is thought to be a cyanide-insensitive pyridine nucleotide-linked oxidase that catalyzes the conversion of oxygen to hydrogen peroxide and superoxide. Major controversy has arisen over which nucleotide is oxidized, and evidence for both NADH and NADPH has been advanced (14–19). There is agreement that the controlling factor for HMPS activity is the availability of NADP+ (20). If the primary oxidase were NADPH-linked, the NADP+ produced would directly stimulate the HMPS. An NADH-linked oxidase would require at least one additional metabolic pathway for the provision of NADP+

(20). In view of the controversy attending this aspect of neutrophil function, we decided to examine the oxidase activity of human PMN, employing resting and phagocytic PMN from normal donors, a group of patients with X-linked CGD, and a patient with myeloperoxidase (MPO) deficiency.

METHODS

Control PMN were obtained from nine adults and two children without CGD, and from one patient with hereditary MPO deficiency. Five of the seven male patients with CGD ranged from 4 to 12 yr of age, and the others were 18 and 28 yr old. Two of the patients were brothers, and all but one, who had Pseudomonas sepsis, were free of serious infection when studied. The diagnosis of CGD was established by measurements of the oxygen consumption, HMPS activity, quantitative iodination, quantitative NBT reduction, and microbicidal activity (Staphlococcus aureus 502 A and Candida albicans) by PMN.

Leukocytes were separated by the Hypaque-Ficoll technique of Boyum (21), and granulocytes were harvested with less than 2% contamination by mononuclear cells. The mean percentages of eosinophils in these preparations were 5.3% (normal controls), 6.8% (MPO-deficient subject), and 6.8% (CGD patients). Erythrocytes were sedimented in dextran, and residual contaminating erythrocytes were lysed by sus-
pending the cell button in cold 0.22% saline for 45 sec and then restoring isotonicity. Granulocytes were centrifuged at 250 g for 10 min and resuspended in Ca**+ and Mg**+-free phosphate-buffered saline (PBS) at a concentration of 1 × 10^6 cells/ml. Zymosan was opsonized by adding AB serum to achieve a concentration of 5 mg/ml, incubating the mixture for 1 min at 37°C., and washing and resuspending the zymosan in PBS at a final concentration of 15 mg zymosan/ml. Phagocytosis was induced by adding 15 mg of zymosan/10^6 PMN, providing approximately 10 zymosan particles/PMN. The cell-zymosan suspension was rotated (30 rpm) at 37°C for 3 min, so that virtually all the neutrophils contained three or more ingested zymosan particles. The reaction was stopped by addition of ice-cold PBS, and the resting and phagocytic cells were immediately centrifuged at 400 g for 3 min at 4°C. The cells were resuspended at a concentration of 5 × 10^6 PMN/ml for 10 min with 2 ml of ice-cold 0.34 M sucrose adjusted to pH 7.4 with NaHCO_3 and homogenized immediately in a motor-driven Teflon-glass homogenizer at 0°C until 95% cell breakage had been achieved. With our homogenizer, 5 min of homogenization was required. After centrifugation at 250 g for 10 min, the supernate was centrifuged at 27,000 g for 20 min to yield a granule-rich sediment and a clear supernate. The granule-rich sediment was resuspended at a concentration of 2 × 10^6 cell equivalents/ml in 0.34 M sucrose, pH 7.4.

Measurements of oxygen consumption were made at 1 mM nucletide concentration at 37°C with a Gilson model KM oximetric system equipped with a Clark-Yellow Springs Instrument oxygen electrode. NADPH oxidase activity was linear with time during the 1st min after addition of enzyme. Reaction rates were calculated from the maximum slope achieved during this period. Nucleotide consumption was monitored as the decrease in absorbance at 340 nm at room temperature in a Beckman Kintrak VII recording spectrophotometer at 110 mm path-length cuvettes. NADPH (98% pure) and NADPH^+ (95-99% pure) were weighed and dissolved in phosphate buffer immediately before use.

The effect of manganese and hydrogen ion concentrations on NADH and NADPH oxidase activity in the 27,000 g supernate was studied by determining the mean enzymatic oxidation of GRF from phagocytic PMN. The corresponding blank values for NADH auto-oxidation were 29% (spectrophotometric assay) and 78% (spectrophotometric assay). Oxidase activity was expressed as nanomoles of O_2 or nucleotide consumed per minute per milligram protein or per 10^6 PMN equivalents. Zymosan caused no intrinsic NAD(P)H oxidase activity, and addition of zymosan to homogenized nonphagocytic PMN caused no increase in NAD(P)H oxidase activity. When PMN were incubated with unopsonized zymosan, there was little microscopic evidence of particle ingestion, and no increase in NAD(P)H oxidase activity was measured.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (protein; Broad). The serum albumin was run as a standard. The total mean protein was 1.31 mg/10^6 PMN (1.49 mg/10^6 PMN) and 1.45 mg/10^6 resting PMN.

**RESULTS**

**Effect of manganese and pH on oxidase activity.** The effects of manganese and pH on oxidase activity in the 27,000 g sediments of phagocytic PMN are shown in Figs. 1 and 2. Oxidase activity with both nucleotides increased 25-fold as the manganese concentration was raised from zero to 0.5 mM. NADPH oxidase was three to six times higher than NADH oxidase at all manganese concentrations and nearly six times higher in the absence of manganese.

When measured in the presence of 0.5 mM manganese, lowering the pH from 7.0-5.5 resulted in a four-fold increase in oxidase activity with both nucleotides: oxidation of NADPH was at least 4.8 times higher than NADH oxidase at all pH values. NAD(P)H oxidase activity of GRF from nonphagocytic cells also varied with Mn**+ concentration and pH; these activities were optimal at pH 5.5 and at a concentration of 0.5 mM Mn**+. The NAD(P)H oxidase activity of GRF...
oxidase activity measured polarographically at 1 mM NAD(P)H concentration in pH 5.5 phosphate buffer. Data are from a representative experiment using the 27,000 g granule-rich sediment of phagocytic PMN as the enzyme source.

varied linearly with the protein concentration over the range employed in our polarographic assays.

In three experiments, we found the apparent $K_m$ for NADPH to be 3.1, 3.3, and $5.7 \times 10^{-5}$ M for GRF from resting PMN and 2.4, 2.5, and $4.0 \times 10^{-5}$ M for GRF from corresponding phagocytic PMN.

Localization of oxidase activity in PMN fractions. The localization of oxidase activity in normal PMN fractions, measured polarographically and expressed as total $O_2$ consumed per minute per $10^7$ PMN equivalents, is shown in Fig. 3. No oxidase activity was detectable with either nucleotide in the whole homogenates of resting PMN, although low levels with both NADPH and NADH were detected in the isolated 27,000 g sediment. This NAD(P)H oxidase activity in GRF from resting PMN was eliminated when granules were suspended in the corresponding 27,000 g supernate rather than in 0.34 M sucrose. Homogenates of phagocytic PMN contained high NADPH oxidase activity; this was localized entirely to the 250 g and 27,000 g sediments, and activity in these fractions was maintained in the presence of the corresponding 27,000 g supernate. NADH oxidase activity in homogenate of phagocytic PMN was substantially lower and also localized to these sediments. We detected no oxidase activity with either NADPH or NADH in the 27,000 g supernate of either resting or phagocytic PMN.

Under the conditions employed, with 1 mM NADPH as the substrate, oxygen consumption by homogenate

![Figure 1](image1.png)

**Figure 1** Effect of manganese concentration on NAD(P)H oxidase activity measured polarographically at 1 mM NAD(P)H concentration in pH 5.5 phosphate buffer. Data are from a representative experiment using the 27,000 g granule-rich sediment of phagocytic PMN as the enzyme source.

![Figure 2](image2.png)

**Figure 2** Effect of pH on NAD(P)H oxidase activity measured polarographically at 1 mM NAD(P)H concentration with 0.5 mM Mn$^{2+}$. Data are from an experiment with the same 27,000 g sediment of phagocytic PMN used in Fig. 1.

![Figure 3](image3.png)

**Figure 3** NAD(P)H oxidase activities in homogenate and fractions of resting (R) and phagocytic (Ø) PMN. Oxidase activity was measured polarographically. Data are from a representative experiment. The dashed line indicates the level of $O_2$ consumption by $10^7$ phagocytic PMN (10 zymosan particles/PMN).
or the combined 250 g and 27,000 g sediments of phagocytic PMN exceeded the increment in oxygen consumption of an equivalent number of intact phagocytic PMN.

**NADPH oxidase activities of controls and patients with CGD.** The specific NADPH oxidase activity of the 27,000 g GRF of resting and phagocytic PMN from controls and the patients with CGD are compared in Fig. 4. In all cases, the 27,000 g fraction of normal phagocytic PMN had higher oxidase activity than the corresponding fraction from resting PMN. Mean NADPH oxidase values from resting and phagocytic PMN, respectively, were 180 and 696 nmol O2/min per mg protein measured polarographically and 103 and 251 nmol NADPH/min per mg protein measured spectrophotometrically. As dissimilar substrate concentrations and temperatures were used in the two assay methods, these values cannot be used to establish the exact stoichiometry of the oxidase reaction.

NADPH oxidase activity was measured in the 27,000 g fraction of PMN from two children, ages 5 and 10, without CGD or serious infection. Resting levels were 85 and 248, and phagocytic levels were 492 and 769. NADPH oxidase activity of comparable fractions from MPO-deficient PMN was 49 (resting) and 367 (phagocytic) nmol O2/min per mg protein. These polarographic values of NADPH oxidase activity were well within the range obtained with PMN from normal adult donors.

Fig. 4 clearly indicates a substantial overlap in NADPH oxidase values between the CGD and control groups when resting PMN were compared. When NADPH oxidase activities of phagocytic PMN were compared, all CGD values were well below the control range, both polarographically and spectrophotometrically. This difference between mean control and mean CGD values was highly significant (P < 0.001) when the data was analyzed by standard t-test. In contrast to the consistent increase in NADPH oxidase activity induced by phagocytosis in normal PMN, leukocytes from all of the patients with CGD lacked significant phagocytic activation.

**NADH oxidase activity of controls and patients with CGD.** The NADH oxidase activity in the 27,000 g sediment of PMN from control donors and patients with CGD is shown in Fig. 5. When measured polarographically in nonphagocytic leukocytes at 1 mM NADH concentration, mean specific NADH oxidase activity was 27 in control PMN and 17.2 in CGD PMN. Although the mean NADH oxidase activity of phagocytic PMN from patients with CGD was lower than the control values (17.7 vs. 87 nmol O2/min per mg protein), there was overlap between individual control and CGD data points. As was the case with NADPH oxidase activity, NADH oxidase activity in PMN from patients with CGD was not activated by phagocytosis. Phagocytic PMN from all controls showed some increase over resting levels of NADH oxidation; the mean increment in NADH oxidase activity was substantially less than that in NADPH oxidase activity (60 vs. 516 nmol O2/min per mg protein).

Low levels of enzyme activity, necessitating large amounts of enzyme, caused difficulties in measuring...
NADH oxidase activity spectrophotometrically at 0.16 mM NADH concentration. There were individual control determinations where no phagocytic increase in NADH oxidase activity was measured; in some of these cases NADH oxidase activity was too low to allow accurate measurement without prohibitively large amounts of enzyme.

**Oxidase activity of PMN from mothers of CGD patients.** NADPH oxidase determinations were made on PMN from mothers of three of the CGD patients. Quantitative NBT reduction and O₂ consumption by PMN from two of these subjects were well below the normal control range; spectrophotometric oxidase activities with NADPH were 83 and 84 (resting PMN) and 145 and 279 (phagocytic PMN). NBT reduction and O₂ consumption by PMN from the third mother were normal; polarographic oxidase activities with NADPH were 11 (resting) and 306 (phagocytic). Additional studies of heterozygotes are required; at present, we are unable to conclude that the GRF from PMN of heterozygous subjects is significantly deficient in NADPH oxidase activity.

**DISCUSSION**

Phagocytosis by mammalian neutrophils is accompanied by marked augmentation of oxidative metabolism. The basic observations of increases in O₂ consumption, glucose oxidation via the hexose monophosphate pathway, and hydrogen peroxide production have been readily confirmed by many investigators. Recognition that PMN from patients with CGD do not show these features of increased oxidative metabolism after ingestion of particles or microorganisms has led to a general appreciation that these metabolic responses are related to microbicidal function. However, controversy has arisen over the particular enzyme(s) primarily responsible for this burst in oxidative metabolism. Clearly, elucidation of the enzymatic basis of the defect in CGD neutrophils could contribute to resolution of the controversy over the primary oxidase of normal neutrophils.

Cagan and Karnovsky (14), and Baehner, Gilman, and Karnovsky (15) have provided evidence for an NADH-linked primary oxidase in the nonphagocytic leukocytes of guinea pigs and man. Although the levels measured were low, these authors reported sufficient oxidase activity in disrupted PMN to account for the burst in oxidative metabolism by intact phagocytic cells. Although Baehner and Karnovsky (23) found abnormally low levels of NADH oxidase activity in the KCl extracts of nonphagocytic leukocytes from five children with CGD, others have been unable to confirm these observations (13).

Evidence for an NADPH-linked primary oxidase has been advanced by a number of investigators, including Iyer and Quastel (18), Rossi et al. (16, 17, 24), and Paul, Strauss, Jacobs, and Sbarra (19). Although Rossi’s group has not reported studies of patients with CGD, they have found a five-fold increase in NADPH oxidase activity with phagocytosis by normal human PMN and have stressed the importance of Mn⁺⁺ and H⁺ concentrations for obtaining optimal enzyme activity (24).

Our own studies have led us to conclude that many of the discrepancies between previous studies of neutrophil oxidase activity are attributable to methodologic differences with major consequences. These include differences related to the H⁺ and Mn⁺⁺ concentrations of the assay mixtures, use of nonphagocytic or phagocytic cells as enzyme sources, and the method of fractionation of the cells to be tested. Each will be commented on briefly.

Our studies offer additional confirmation to the generally held belief that pyridine nucleotide oxidase activity is optimal at acid pH levels (9, 17, 19), yet some investigators have chosen to perform measurements at suboptimal pH levels (13-15, 23). This has reduced the sensitivity of their measurements and necessitated extrapolation to presumed optimal pH values to determine whether the measured enzyme levels would suffice to account for the magnitude of the metabolic stimulation of an equivalent number of phagocytic cells (23).

Considerable importance attends the use of Mn⁺⁺ in assays of oxidase activity. We found that addition of a variety of other cations, including Mg⁺⁺, Ca⁺⁺, Fe⁺⁺, Fe⁺⁺⁺, Cu⁺⁺, and Zn⁺⁺, caused no increase in NADP⁺H oxidase activity. Iyer and Quastel noted the Mn⁺⁺-stimulated oxidation of NADH and NADPH by oxidases of guinea pig PMN (18); Roberts and Quastel later attributed this effect, in part, to a Mn⁺⁺-dependent oxidation of these nucleotides by MPO (25, 26). However, several considerations indicated that Mn⁺⁺ exerts an effect on NAD(P)H oxidase activity that is completely independent of MPO. Rossi, Romeo, and Patriarca found that Mn⁺⁺ stimulated the oxidase activity of MPO-deficient neutrophils (24), a finding also confirmed in the present study. We have found that neutrophils from subjects with CGD have low levels of oxidase activity in the presence of Mn⁺⁺, despite their normal levels of MPO. The failure of MPO to obscure these measurements of NAD(P)H oxidase activity may be attributed to the presence of 2 mM KCN in the mixtures used to assay oxidase activity. We observed that this concentration sufficed to inhibit completely the Mn⁺⁺-dependent oxidation of both nucleotides by purified human MPO.⁷

We do not yet know if the Mn⁺⁺ effect on NAD(P)H oxidase activity is expressed in intact cells. Although

⁷ Generously provided by Dr. J. Schultz.
neutrophils are rich in zinc, we are aware of only one report dealing with their content of manganese (27). Atomic absorption spectrophotometric studies obtained on neutrophils fractionated by the methods employed in this study revealed Mn content in the 27,000 g GRF of normal neutrophils, where the metal was found to be 7.9% as abundant on a molar basis as zinc. The 27,000 g supernate had no detectable Mn. Although additional studies will be required to ascertain the functional significance of human neutrophil Mn, its presence and relative abundance in the oxidase-containing fraction suggest that its role in NAD(P)H oxidase activity may be more than an interesting in vitro phenomenon.

The selection of phagocytic versus nonphagocytic cells for measurements of oxidase activity is of critical significance. We found no consistent difference between normal and CGD neutrophils when we examined nonphagocytic cells. Yet clear and consistent differences in NADPH oxidation were apparent when phagocytic cells were compared. In preparing phagocytic PMN for our studies, we selected conditions that induced a rapid and maximal stimulation of O2 consumption and made our enzyme measurements on cells fractionated soon after attaining rates of O2 consumption some 15–20 times their basal levels. In the only other study that has employed phagocytic PMN from patients with CGD, Baehner and Nathan stimulated the cells with polystyrene spheres or bacteria for 15 min, and granules were lysed by repeated cycles of freezing and thawing (12). In our hands, both prolonged incubation (more than 7 min) of cell-particle suspensions and freeze-thaw treatment resulted in major losses of enzyme activity.

A final important methodologic point relates to the fraction in which enzyme activity was measured. We found essentially no activity in the 27,000 g cytosol after homogenization of the cells in 0.34 M sucrose. Activity was localized to the 250 g and 27,000 g sediments, both of which contained large numbers of granules as shown by morphology and by measurements of marker enzymes; oxidase activity of GRF was completely abolished by boiling and by incubation with pronase and trypsin. Our attempts to solubilize the oxidase by extraction of GRF with dilute acids or isotonic or hypertonic KCl resulted in considerable loss of activity. We preferred to make our measurements on the 27,000 g GRF, a reasonably consistent enzyme source that was not unduly influenced by variations in the extent of successful cell disruption. It should be noted that Rossi et al. had earlier recommended the use of this fraction.

Baehner and Karnovsky reported that NADH oxidase activity in 30,000 g alkaline KCl supernate of nonphagocytic neutrophils was lower in five children with CGD than in controls (23). It appears, however, that the controls were patients who may have had leukocytosis associated with infection (28). Holmes, Page, and Good did not confirm these differences with normal uninfected subjects as controls (13). Baehner and Karnovsky reported that NADPH oxidase in neutrophil granules was approximately one-sixth as active as the NADH oxidase of normal neutrophils. Their measurements were made on nonphagocytic PMN, at pH 7, in the absence of manganese. NADPH oxidase activity was not reported in phagocytic cells, in patients with CGD, or under optimal conditions of assay. Baehner and Nathan found lower levels of NADPH oxidase activity in supernates of phagocytic cells from two children with CGD than in normal controls (12). They found little NADPH oxidase activity in the granule fraction of normal cells and found no NADPH oxidase activity in either granules or supernate. Their granules, however, were subjected to repeated freezing and thawing, a treatment that would markedly decrease NADPH oxidase activity. Furthermore, NADPH oxidase was measured at pH 7.0 and in the absence of Mn++, resulting in further enzyme attenuation.

Although we maintain reservations concerning the validity of comparing oxidase activity in disrupted cell fractions with levels of O2 consumption by intact leukocytes, our studies indicate that, when NADPH served as the substrate, O2 consumption by homogenized cells considerably exceeds the level of O2 utilization by maximally phagocytic PMNs. These high levels of total and specific NADPH oxidase activity were observed only in homogenates of normal phagocytic neutrophils; nonphagocytic PMNs had much lower oxidase activity.

Our studies revealed that nonphagocytic PMN from patients with CGD had mean oxidase activities with both NADPH and NADH that were somewhat lower than corresponding mean values of the control group; however, overlap between data points was substantial, and separation of control and CGD groups was incomplete. The mean values of NADH oxidase activity of phagocytic PMN from patients with CGD were lower than the corresponding control values, but the ranges overlapped. Although none of the aforementioned measurements allowed us to separate CGD from normal PMN, our data show a marked difference when the NADPH oxidase activities of phagocytic PMN are compared. Phagocytic neutrophils from seven patients with CGD were deficient in NADPH oxidase activity, with wide separation between patient and control groups. Moreover, PMN from none of the CGD patients showed the activation of NADPH oxidase ac-
tivity by phagocytosis that was consistently observed with control PMN. We believe that these data indicate that absence of a cyanide-insensitive NADPH oxidase or deficiency in its activation is the primary metabolic defect in the X-linked CGD syndrome.

Attention should now be directed to a number of important problems that remain. These include determining whether NADPH and NADH are oxidized by the same or separate oxidases, precise localization of the oxidase(s), isolation and purification of the enzyme(s), and elucidation of the mechanism and effectors involved in the activation of oxidase activity by the phagocytic event.

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