Deficient Flavoprotein Component of the NADPH-dependent O_2^- -generating Oxidase in the Neutrophils from Three Male Patients with Chronic Granulomatous Disease

Theodore G. Gabig and Bruce A. Lefker

Department of Medicine, University of Michigan School of Medicine and the Ann Arbor Veterans Administration Hospital, Ann Arbor, Michigan 48105

Abstract. The NADPH-dependent O_2^- -generating oxidase in subcellular fractions from the neutrophils of three male patients with chronic granulomatous disease was compared with the corresponding preparations from normal neutrophils. The oxidase from normal neutrophils contained flavin adenine dinucleotide in an $\sim 0.9:1$ molar ratio with cytochrome b_{559} . Each of the three chronic granulomatous disease patients had decreased amounts of the flavoprotein component of the oxidase fraction. The oxidase from two chronic granulomatous disease patients had undetectable amounts of cytochrome b_{559} whereas the third patient had a normal content of cytochrome b_{559} , which was spectrally indistinguishable from the normal. The intrinsic cytochrome b_{559} in the oxidase fraction from stimulated neutrophils of the latter chronic granulomatous disease patient was not reduced by NADPH under anaerobic conditions, in distinction with the previously reported reduction of the normal cytochrome b_{559} under identical conditions. We conclude that the flavoprotein component of the oxidase may mediate transfer of electrons from NADPH to the cytochrome b_{559} in normal neutrophils, and that deficiency of this flavoprotein is associated with the chronic granulomatous disease phenotype in the three patients studied.

Introduction

Human neutrophils contain an oxidase system capable of killing ingested microorganisms by delivering potent oxidant species into the phagolysosome (1, 2). Control of oxygen activation for

this process is mediated by an activatable NADPH-dependent (3, 4) O_2^- -generating oxidase that requires flavin adenine dinucleotide (FAD)¹ (5, 6) as an essential cofactor. The overall reaction catalyzed by this system is: $NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2H^+ + 2O_2^-$. Chronic granulomatous disease (CGD) is a genetically transmitted disorder characterized by severe and recurrent bacterial infections resulting from a defect in this oxidase system (7). Evidence that a unique cytochrome b is an essential component of this system is supported by the study of certain CGD patients. Cytochrome b_{-245} (designated cytochrome b_{559} herein to reflect its α -band absorption at 559 nm) was undetectable in the neutrophils from most males with the X-linked form of CGD (8). The cytochrome b_{-245} was present but failed to become reduced following stimulation of intact neutrophils from certain patients with the autosomal recessive form of the disease (8, 9). We have recently reported a flavoprotein component of the subcellular NADPH dependent O_2^- -generating oxidase from normal neutrophils that was present in a 0.9:1 molar ratio to the cytochrome b_{559} and could be completely resolved from the cytochrome b_{559} component (10). The flavoprotein component was undetectable in the resolved oxidase fraction from the neutrophils of one male patient with CGD that had a normal component of cytochrome b_{559} . The present study demonstrates that deficiency of this flavoprotein is associated with the failure of NADPH to reduce the intrinsic cytochrome b_{559} in the subcellular particulate oxidase fraction prepared from the stimulated neutrophils of this patient. Two additional male patients with CGD are reported whose neutrophils were deficient in both flavoprotein and cytochrome b_{559} components.

Methods

Cytochrome c (horse heart, type VI), superoxide dismutase (bovine erythrocyte), NADP, reduced form (NADPH, preweighed vials), deoxycholic acid, cholic acid, and phorbol myristate acetate were obtained from Sigma Chemical Co., St. Louis, MO. Lymphoprep was obtained

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1. Abbreviations used in this paper: CGD, chronic granulomatous disease; FAD, flavin adenine dinucleotide; NBT, nitroblue tetrazolium.

from Accurate Chemical & Scientific Corp., Westbury, NY. Helium (99.9999%) and a room temperature catalytic gas purifier model 4506 were obtained from Matheson, Div., Searle Medical Products, Secaucus, NJ. Deoxycholic acid or cholic acid in hot 50% (vol/vol) ethanol solutions, were absorbed with activated charcoal, recrystallized, then brought to 10% (wt/vol) solution, pH 8.4 (deoxycholate) or 20% (wt/vol) solution, pH 8.2 (cholate) by neutralization with potassium hydroxide (11).

CGD patients. Blood for these studies was drawn after informed consent from the patient (parent or legal guardian of minors) was obtained in accordance with guidelines set forth by the Department of Health and Human Services and local Institutional Review Board. M.K., a 22-yr-old male, referred to as CGD₂ in a previous report (10) has had recurrent *Staphylococcus aureus* skin, pulmonary, lymph node, and hepatic abscesses since the age of ~1.5 yr. He is the only known clinically affected member of the kindred. M.K.'s mother, father, and sole sibling, a sister, had a normal pattern on the stimulated nitroblue tetrazolium (NBT) slide test (18). J.C., a 14-yr-old male, has had recurrent suppurative lymphadenitis and superficial *S. aureus* skin infections since the age of 2. He was adopted at 3 wk of age and no information is available about his biologic family. R.B., an 11-mo-old male, was recently diagnosed, after being evaluated for lymphadenopathy, recurrent vomiting, and failure to thrive. One maternal uncle had a history of recurrent pneumonia and died of overwhelming bacterial sepsis at 50 yr of age, one maternal male first cousin died of bacterial pneumonia and sepsis at 2 yr of age. No clinically affected females were known in this kindred, whereas the mother and maternal aunt both had an abnormal mosaic pattern of NBT-positive and -negative cells on the stimulated NBT slide test (21). R.B. and J.C. have not been reported previously.

Isolation, stimulation, and fractionation of neutrophils. Neutrophils from normal volunteers or CGD patients were isolated from fresh blood (12), stimulated with phorbol myristate acetate, sonically disrupted, and then fractionated by differential isopycnic sedimentation identically to a previously reported method (13). The particulate fraction from this procedure was enriched in cytochrome *b*₅₅₉ content and NADPH-dependent O₂⁻-generating activity and was used as the starting material for the bile salt fractionation.

Bile salt fractionation. The particulate oxidase fraction from the above step was resolved into flavoprotein and cytochrome *b*₅₅₉ components by selective bile salt extraction identically to a previously reported method (10).

Anaerobic methods. Anaerobic conditions were achieved in specially designed 1-cm path length Thunberg type fluorometric or spectrophotometric cuvettes by cyclic evacuation and equilibration with purified helium (99.9999%) from a gas train after the design of Beinert et al. (14). The details of this system have been reported in detail (13).

Spectrophotometric and spectrofluorometric methods. All spectrophotometric measurements were performed in a Cary 219 spectrophotometer (Varian Associates, Inc., Instrument Group, Palo Alto, CA) equipped with automatic programmable base-line and thermostatted cuvette holders. All spectrofluorometric measurements were performed on a Perkin Elmer MPF 44B spectrofluorometer (Perkin-Elmer Corp., Physical Electronic Div., Eden Prairie, MN) equipped with a thermostatted cuvette holder and a differential corrected spectra (DCSU II) unit. All fluorescent spectra were corrected for intensity of excitation light. Reduction of the intrinsic cytochrome *b*₅₅₉ by NADPH was performed under anaerobic conditions identically to a previously reported method (13), except for the addition of FAD (2 μM final) in certain control experiments.

Analysis of cytochrome *b*₅₅₉, FAD and flavoprotein. Neutrophil subcellular fractions were analyzed for content of cytochrome *b*₅₅₉ by dif-

ference absorption spectroscopy following complete anaerobic reduction by dithionite (13) utilizing a reduced minus oxidized (559–540 nm) extinction coefficient of 21.6 mM⁻¹ cm⁻¹ (15). The same subcellular fractions were analyzed for content of FAD quantitatively (16) and flavoprotein qualitatively by spectrofluorometric methods. Protein concentration was determined by a dye binding assay (Bio-Rad Laboratories, Richmond, CA) (17).

O₂⁻ production. Superoxide production by intact cells (4) or subcellular neutrophil fractions (13) was assayed identically to previously reported methods.

Results

Stimulated neutrophils from each of the CGD patients failed to reduce NBT (18) and produced no detectable amounts of O₂⁻ when stimulated by serum opsonized zymosan or phorbol myristate acetate in amounts causing maximal stimulation of normal neutrophils (4). The subcellular particulate oxidase fraction from stimulated neutrophils obtained from each of the patients was compared with the counterpart from normal neutrophils. Table I compares the content of FAD and cytochrome *b*₅₅₉ in these particulate preparations. The particulate oxidase fraction from normal neutrophils contained FAD and cytochrome *b*₅₅₉ in an ~0.9:1 molar ratio. Neutrophils from CGD patients J.C. and R.B. had ~20 and 12%, respectively, of the normal amount of FAD and no detectable cytochrome *b*₅₅₉ in the particulate oxidase fraction. Neutrophils from CGD patient M.K. had a normal content of cytochrome *b*₅₅₉ and <10% of the normal amount of FAD in the particulate oxidase fraction.

The resolved flavoprotein fractions from the oxidase of CGD patients M.K. and J.C. were compared with the normal resolved flavoprotein. Fig. 1 shows the fluorescence excitation spectra of these preparations. The resolved flavoprotein from normal neutrophils displayed excitation maxima at 375 and 450 nm when emission was monitored at 530 nm (10), characteristics that are typical of certain oxidized flavoproteins (19). Fluorescence excitation spectra of the corresponding oxidase fractions from patients M.K. and J.C. had a low level of fluorescence and no

Table I. FAD and Cytochrome *b*₅₅₉ Content of the Particulate Neutrophil Oxidase

Particulate neutrophil oxidase	FAD	Cytochrome <i>b</i> ₅₅₉
	nmol/mg protein	nmol/mg protein
Normal (<i>n</i> = 8)	0.25±0.02	0.28±0.01
CGD _{jc} (<i>n</i> = 3)	0.05±0.01	<0.01
CGD _{rb} (<i>n</i> = 1)	0.03	<0.01
CGD _{mk} (<i>n</i> = 3)	<0.02	0.27±0.01

Data represent mean±SE. The subcellular particulate NADPH-dependent O₂⁻-generating oxidase fraction was prepared from the stimulated neutrophils of normal volunteers or one of three CGD patients (CGD_{jc}, CGD_{rb}, and CGD_{mk}) and then analyzed for content of FAD and cytochrome *b*₅₅₉ identically to a previously reported method (10).

Figure 1. Fluorescence excitation spectra of the resolved flavoprotein fraction. The resolved flavoprotein fraction was prepared from the stimulated neutrophils of normal volunteers or one of two male patients with CGD identically to the previously reported method (10). Under aerobic conditions, excitation spectra were performed (slit width 5 nm) while emission was monitored at its maximum of 530 nm (slit width 10 nm) (10). At the identical gain setting, the spectrum of a sample prepared from normal neutrophils (normal, 1.2 mg protein/ml) or from patients with CGD (CGD_{jc}, 1.6 mg protein/ml and CGD_{mk}, 1.5 mg protein/ml) was as indicated.

sharply resolved maxima. Due to R.B.'s age and small size, sufficient material could not be obtained to study the resolved flavoprotein fraction from his neutrophils.

Control experiments were performed to exclude the possibility that a material was present in the bile salt fraction from CGD neutrophils (corresponding to the flavoprotein fraction from normal cells) that quenched or otherwise interfered with the qualitative flavoprotein spectra. In these experiments, the resolved flavoprotein fraction from normal neutrophils was mixed with the corresponding fraction from the neutrophils of J.C. The fluorescence excitation spectra of these mixtures were additive (Fig. 2) indicating that significant quenching of the normal flavoprotein spectrum did not account for the qualitatively abnormal flavoprotein spectrum of J.C. Separate control experiments were performed on the oxidase fraction from M.K.'s neutrophils to determine if a substance was present that interfered with the tricarboxylic acid extraction and quantitative determination of FAD. In these experiments authentic FAD or an equal amount of normal control particulate oxidase protein was mixed with the particulate oxidase fraction from M.K.'s neutrophils. Subsequent quantitative analysis of these mixtures for FAD resulted in complete recovery of the expected amount of FAD, indicating that the low FAD content of M.K.'s oxidase fraction (before bile salt resolution) was not due to interference with the assay.

The resolved cytochrome b_{559} fraction from the particulate oxidase of the CGD patients was compared with the normal resolved cytochrome b_{559} . Fig. 3 shows the anaerobic dithionite fully reduced minus oxidized absorption spectrum of each of the CGD patients. The spectrum of the resolved cytochrome b_{559} from M.K. was similar to the previously published spectrum of the particulate-bound cytochrome b_{245} (22), and nearly identical to that of the resolved cytochrome b_{559} from normal neutrophils (10). The spectra of the corresponding oxidase fraction from the neutrophils of patients J.C. and R.B. had no absorption characteristics that resembled the normal cytochrome b_{559} . The absorbance maximum at 474 nm may represent contaminating myeloperoxidase in these preparations.

Since the cytochrome b_{559} in the neutrophil oxidase fraction from patient M.K. was spectrally identical to normal, we studied the reduction of the intrinsic cytochrome b_{559} by NADPH in this preparation. We have previously reported that the intrinsic cytochrome b_{559} in the oxidase fraction prepared from normal stimulated neutrophils was reduced by NADPH under anaerobic conditions but failed to undergo reduction under identical conditions in the oxidase fraction from unstimulated cells (13). Table II compares reduction of the intrinsic cytochrome b_{559} in the oxidase fraction prepared from the stimulated neutrophils of patient M.K. to that in the oxidase fraction prepared from normal stimulated neutrophils. No detectable reduction of the cytochrome b_{559} from M.K. occurred at 2 min or 2 h after the anaerobic addition of 100 μ M NADPH whereas the normal cytochrome b_{559} underwent $\sim 20\%$ (2 min) and 45% (2 h) reduction under the same conditions.

Figure 2. Fluorescence excitation spectra of mixed normal and CGD flavoprotein fractions. Resolved flavoprotein fractions from a normal control and patient J.C. were prepared and spectra recorded as in Fig. 1. The excitation spectra of the normal flavoprotein (—), the corresponding fraction from J.C. (---), or mixtures of these fractions (---) were recorded at the same gain setting. The proportion of normal/normal plus J.C. (vol/vol) is indicated above the appropriate spectra of the mixtures.

Figure 3. Dithionite reduced minus oxidized absorption spectra. The fraction corresponding to the resolved cytochrome b_{559} fraction from normal stimulated neutrophils was prepared from stimulated neutrophils from each of the CGD patients as reported (10). Under anaerobic conditions, dithionite fully reduced minus oxidized difference spectra were recorded of the preparations (CGD_{jc}, 0.9 mg protein/ml, CGD_{mk}, 0.4 mg protein/ml, CGD_{rb}, 1.4 mg protein/ml) identically to the previously reported method (13).

These experiments were repeated to determine if added FAD or higher NADPH concentrations could force the reduction of M.K.'s cytochrome b_{559} . Experiments were performed in the dark following FAD addition. In the presence of added FAD, (2 μ M) no detectable reduction of M.K.'s intrinsic cytochrome b_{559} occurred 2 min after anaerobic NADPH addition (100 μ M), however, $\sim$ 10% of M.K.'s intrinsic cytochrome b_{559} was reduced at 20 min. Increasing the NADPH concentration to 300 μ M at 20 min resulted in no additional reduction of M.K.'s cytochrome b_{559} over the subsequent 20 min. No reduction of the added FAD was observed during this experiment with M.K.'s oxidase. Control experiments performed on particulate oxidase fractions from normal, stimulated neutrophils in the presence of 2 μ M FAD differed from those performed on M.K.'s oxidase with added FAD as well as from those on the normal oxidase in the absence of FAD. In the presence of 2 μ M FAD, the rate of normal cytochrome b_{559} reduction by NADPH was slower and more variable, reaching only $12.5 \pm 2.3\%$ (mean $\pm$ SE, $n = 4$) 20 min after anaerobic addition of 100 μ M NADPH. Additionally, intrinsic cytochrome b_{559} reduction was accompanied by simultaneous reduction of the added FAD, this reduction being complete at 20 min after addition of NADPH.

Experiments were performed in attempts to reconstitute the oxidase fraction from M.K.'s neutrophils with the resolved fla-

voprotein fraction from normal neutrophils and/or FAD. As reported previously (23), recombining the resolved flavoprotein and cytochrome b_{559} fractions from normal stimulated neutrophils in the presence of sonicated phosphatidylethanolamine (1.25 mg/ml) and FAD (20 μ M) resulted in partial reconstitution ($\sim$ 3–6%) of NADPH-dependent O_2^- -generating activity. An experiment in which the particulate oxidase fraction from M.K.'s stimulated neutrophils was combined with the resolved flavoprotein fraction from normal stimulated neutrophils under the latter conditions failed to reconstitute measurable NADPH-dependent O_2^- generation. Additionally, FAD alone (60 μ M in assay) failed to restore NADPH-dependent O_2^- generation by the particulate oxidase from M.K.'s stimulated neutrophils.

Discussion

The three patients with CGD reported in the present study lacked normal amounts of the flavoprotein component of the neutrophil oxidase fraction. Each of the patients were males whose neutrophils were incapable of generating detectable amounts of O_2^- or reducing detectable amounts of NBT when stimulated. The patients differed, however, in that neutrophils from patient M.K. contained normal amounts of the cytochrome b_{559} , whereas those from the other two were lacking this cytochrome. In patient M.K., deficiency of the flavoprotein component was associated with the inability of NADPH to reduce the intrinsic cytochrome b_{559} in the particulate oxidase from his stimulated neutrophils. This NADPH-dependent reduction of the endogenous cytochrome b_{559} occurred in corresponding preparations from stimulated normal neutrophils. The finding that added FAD allows NADPH-dependent reduction of the intrinsic cytochrome b_{559} in M.K.'s oxidase is not conclusive evidence that his cytochrome b_{559} is functionally normal, since neither FAD nor the normal resolved flavoprotein were capable

Table II. Reduction of the Endogenous Cytochrome b_{559} by NADPH

Neutrophil oxidase	NADPH-dependent cytochrome b_{559} reduction	
	2 min	2 h
	% total, mean $\pm$ SE	% total, mean $\pm$ SE
Normal ($n = 6$)	20 ± 4	45 ± 6
CGD _{mk} ($n = 3$)	<1	<1

The NADPH-dependent O_2^- -generating oxidase fraction was prepared from stimulated neutrophils of normal volunteers or a male patient with CGD (CGD_{mk}) and then analyzed for NADPH-dependent reduction of the cytochrome b_{559} under anaerobic conditions identically to the previously described method (13). Reduction of the cytochrome b_{559} at the indicated times following anaerobic addition of NADPH is reported as the percentage of total cytochrome b_{559} present in the preparation (determined separately).

of restoring O_2^- -generating activity. Indeed the effect of added FAD on reduction of the intrinsic cytochrome b_{559} in the normal oxidase suggests that reduction of added FAD may be a competing enzymatic or nonenzymatic reaction in the normal preparation. These observations suggest that the flavoprotein component of the oxidase fraction from stimulated normal neutrophils mediates electron transfer from NADPH to the cytochrome b_{559} . We postulate that the CGD phenotype in this patient is a result of deficiency of the flavoprotein component of the NADPH-dependent O_2^- -generating oxidase. However, an intrinsic functional abnormality of the cytochrome b_{559} in patient M.K. has not been excluded.

Patients J.C. and R.B. were deficient in both flavoprotein and cytochrome b_{559} components of the oxidase. Therefore, NADPH-dependent reduction of the endogenous cytochrome b_{559} could not be studied in these patients. The seeming abnormality of two polypeptides resulting from a presumed single mutation in the latter patients is not easily explained by our current understanding of the disease and the normal oxidase enzyme. A variety of possible explanations exist at levels of gene expression, transcription, or translation to explain the deficiency of both components in these patients. Cross et al. (20) have recently reported the association of FAD with the cytochrome b_{-245} from normal neutrophils and found ~50% of the normal level of FAD in the corresponding neutrophil fraction from a male CGD patient with absent cytochrome b_{-245} . Until further purification and characterization of both flavoprotein and cytochrome b_{559} components is accomplished, explanation of the dual abnormalities in these patients must remain speculative.

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