Absence of Both the 91kD and 22kD Subunits of Human Neutrophil Cytochrome \( b \) in Two Genetic Forms of Chronic Granulomatous Disease

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Chronic granulomatous disease (CGD) is a group of inherited disorders in which phagocytic cells fail to generate antimicrobial oxidants. The various forms of CGD can be classified in terms of the mode of inheritance (either X-linked or autosomal recessive), and whether the neutrophils display the absorbance spectrum of a unique b-type cytochrome important for the function of the respiratory burst oxidase. The finding that purified neutrophil cytochrome \( b \) is a heterodimer consisting of a 91kD glycosylated and a 22kD nonglycosylated polypeptide has raised the question of which subunits are absent (or defective) in the various types of CGD. To address this question we have studied the expression of the cytochrome \( b \) subunits in three genetically distinct forms of CGD: X-linked/cytochrome \( b \)-negative (X\(^-\)), autosomal recessive/cytochrome \( b \)-negative (A\(^-\)), and autosomal recessive/cytochrome \( b \)-positive (A\(^+\)). Using polyclonal antibodies to each of the two subunits, we prepared Western blots of lysates of intact neutrophils from ten CGD patients. In the controls and three patients with A\(^-\) CGD, both cytochrome subunits were easily detected. Consistent with the previously reported finding in five X\(^-\) patients, neither subunit could be identified in neutrophils from three additional X\(^-\) patients. Both subunits were also undetectable in four patients with A\(^+\) CGD (three females, one male). This latter group of patients most likely bears a normal 91kD gene, since the patients are genetically distinct from the 91kD-defective X\(^+\) group. The mutation in A\(^+\) CGD, therefore, probably involves the 22kD gene and the eventual expression of the 22kD subunit. Furthermore, the expression of the 91kD subunit in this group of patients appears to be prevented due to the 22kD mutation in a manner converse to that seen in the X\(^-\) CGD patients. Based on these studies, we hypothesize that the stable of expression of either of the two cytochrome subunits is dependent upon the other.

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Chronic granulomatous disease (CGD) is an inherited disorder of host defense in which neutrophils and other phagocytic cells fail to generate superoxide (O\(_2^-\)) and hydrogen peroxide upon stimulation. Since these reactive oxygen compounds are required for the killing of many microorganisms, CGD patients suffer from recurrent and sometimes life-threatening infections. The enzyme responsible for O\(_2^-\) production in human neutrophils is nicotinamide adenine dinucleotide phosphate, reduced (NADPH) oxidase, a membrane-bound flavoprotein complex that is catalytically inactive in unstimulated cells, but becomes activated following exposure of neutrophils to such stimuli as opsonized microbes, chemotactic peptides, and a variety of other agents. Besides a group of at least four cytosolic proteins and putative additional membrane components, it is believed that a low-potential \( b \)-type cytochrome is also part of the active enzyme and that it is responsible for the reduction of oxygen to O\(_2^-\). The activity of this NADPH oxidase is absent or greatly diminished in CGD neutrophils.

A central role for cytochrome \( b \) in oxidase function has been supported by the finding that it is spectrally absent in some forms of CGD. In both the classic X-linked form of the disease and a rare subset of the autosomal recessive form, the cytochrome \( b \) spectrum cannot be detected in neutrophils. On the other hand, the majority of the autosomal recessive patients have normal levels of cytochrome \( b \). This heterogeneity in both the mode of inheritance and the presence of the cytochrome \( b \) spectrum suggests that at least several distinct genetic mutations can result in the phenotype of CGD. This is supported by genetic complementation studies using CGD monocyte hybrids that revealed three major complementation groups in CGD, X-linked/cytochrome-negative (X\(^-\)), autosomal recessive/cytochrome-negative (A\(^-\)), and autosomal recessive/cytochrome-positive (A\(^+\)).

The importance of cytochrome \( b \) in CGD has been unequivocally established by the application of research involving the purification of the cytochrome and studies on the molecular genetics of the X-linked form of CGD. Human neutrophil cytochrome \( b \) has been purified and shown to consist of two subunits: a heavily glycosylated polypeptide with an M\(_r\) of 91kD, and a smaller, nonglycosylated 22kD polypeptide. The gene, which is defective in X-linked CGD, has been cloned and shown to encode for the 91kD cytochrome subunit. The 22kD subunit has also been cloned and been shown to be absent in the X\(^-\) form of CGD. Thus, the expression of the 22kD subunit of cytochrome \( b \) appears to be somehow linked to the expression of the 91kD subunit.

Two major questions are raised by these studies with
regard to CGD. First, does the genetic absence of the one subunit of the cytochrome in X-linked CGD consistently affect the expression of the other subunit? Second, in the other autosomal forms of CGD, how are the two cytochrome subunits affected? In this report, we have studied the cytochrome b subunit composition in the three major genetic forms of CGD using antibodies directed against each of the subunits. Our results indicate that the genetic absence of one of the cytochrome subunits is accompanied by the absence of the other in both the X- and A- forms of CGD, but not the A+ type.

MATERIALS AND METHODS

Patients. Three patients with X- CGD, not previously analyzed by Western blot analysis for cytochrome b subunits, were studied. All three had undetectable levels of cytochrome b by difference spectroscopy. Patients E.H. and J.C. have been previously reported.23 The mother and sister of patient M.P. are carriers of CGD based on a dimorphic staining pattern in the nitroblue tetrazolium slide test.14 Three patients with A- CGD were studied, all of whom have been previously reported:18 N.S., R.G., and J.C. Three females and one male with A- CGD were examined in this study.

Preparation of antibodies to cytochrome b. Rabbit antiserum reactive with the Mr = 22,000 light chain of cytochrome b has been described elsewhere.9 To obtain antibodies reactive with the Mr = 91,000 heavy chain of the cytochrome, both a β-galactosidase-91kD fusion protein and synthetic peptides derived from the sequence of the heavy chain described by Royer-Pokora et al19 and Teahan et al25 were used as antigens. The rabbit antiserum raised against the former has been previously described.21 Antisera to the latter were raised against two cysteine-conjugated peptides, ARKRIKN-PEGGC (Residues 154 through 164) and KQSIENSINSPGRGC (Residues 546 through 558), which were kindly prepared by Dr Michael Buchmeier (Research Institute of Scripps Clinic, La Jolla, CA) as described previously.26 The peptides were coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde as previously described, substituting KLH for albumin.27

Rabbits were initially immunized intradermally with 1 mg of the peptide-KLH conjugate emulsified with complete Freund's adju-

![Western blots of X and A CGD neutrophils with antibodies to both subunits of cytochrome b.](image-url)

Fig 1. Western blots of X- and A- CGD neutrophils with antibodies to both subunits of cytochrome b. Antibodies to the Mr = 91,000 heavy chain (upper panel) and Mr = 22,000 (lower panel) of cytochrome b were used to probe neutrophils from patients with X- and A- CGD. The corresponding lanes in both panels contain the same samples as follows: lane 1, 1 μg purified cytochrome b; lanes 2 through 4, normal human neutrophils; lane 5, neutrophils from an X- CGD carrier (female) which had a diminished ability to produce superoxide anion (25% of normal); lanes 6 through 8, neutrophils from three male X- CGD patients; lanes 9 through 11, neutrophils from three female A- CGD patients. Lanes 2 through 11 each contained 40 μg of protein from a Triton X-100 lysate of intact neutrophils (equivalent to 1.5 to 2.5 x 10⁶ cells) that was prepared as described in Materials and Methods.
vant. Intradermal booster injections with the same amount of KLH-peptide emulsified in incomplete Freund’s adjuvant were given at 2, 4, and 6 weeks, respectively. Antiserum obtained 1 week after the last booster injection was assayed for reactivity with cytochrome b by Western blot analysis.

Sample preparation and Western blotting. Neutrophils were prepared as previously described, treated with diisopropyl fluorophosphate, and frozen at -70°C in aliquots containing 5 x 10^6 PMN. Neutrophils and cytochrome b samples were prepared for Western blotting as described previously.

RESULTS

Because of the dual subunit structure of the cytochrome b, we investigated the possibility that each of the two cytochrome-negative forms of CGD might be due to a defect in one or the other subunits of the cytochrome in a complementary fashion. To test our hypothesis, we examined the reactivity of several anticytocrome antibodies with both purified cytochrome b and extracts from normal and CGD patient neutrophils. The pooled antisera prepared against peptide regions of the 91kD subunit reacted strongly with the M, = 91,000 heavy chain of both purified cytochrome b and normal neutrophil extracts on Western blots as shown in the upper panel of Fig 1, lanes 1 and 2 through 4, respectively. The other bands in the neutrophil lanes are the result of nonspecific antibody labeling, since competition experiments with excess synthetic peptides did not inhibit their labeling while that of the 91kD band was completely blocked (data not shown). Similar results were obtained using the anti-91kD β-galactosidase fusion protein antiserum (data not shown).

Lanes 6 through 8 show the reactivity of the pooled anti-91kD peptide antibodies to neutrophil extracts from three males with X- CGD and show complete absence of the broad band characteristic of the 91kD subunit. This observation confirms the previously reported finding using antisera directed against the 91kD fusion protein. Lane 5 shows an intermediate level of 91kD subunit in a neutrophil extract from a female carrier of X- CGD who had only 25% normal respiratory burst activity. Lanes 9 through 11 are from patients with autosomal recessive cytochrome-positive CGD (A+), in whom the cytosol factor activity required for oxidase activation is abnormal. These cells contain normal levels of cytochrome b as determined by absorbance difference (reduced minus oxidized) spectroscopy and show normal labeling of the 91kD subunit. The lower panel of Fig 1 examines the content of the 22kD subunit of cytochrome b in the same extracts analyzed in the upper panel. As can be seen, the amount of this subunit parallels the levels of the 91kD subunit observed in the upper panel. The result is noteworthy in that all three X- CGD patients whose defect resides in the 91kD gene are obviously also deficient in the 22kD subunit.

![Antisera to N- and C-termini peptides from the 91 kD subunit](image1)

![Antiserum to the 22 kD subunit](image2)

**Fig 2.** Western blots of A- CGD neutrophils with antibodies to both subunits of cytochrome b. Antibodies to the M, = 91,000 heavy chain (upper panel) and M, = 22,000 light chain (lower panel) of cytochrome b were used to probe neutrophils from patients with A+ CGD. The corresponding lanes in both panels contain the same samples as follows: lane 1, 1 μg purified cytochrome b; lanes 2 and 3, normal human neutrophils; lanes 4 through 7, neutrophils from four patients (one male, three females) with A- CGD. Lanes 2 through 7 contained 40 μg of protein from a Triton X-100 lysate of intact neutrophils (1.5 to 2.5 x 10^6 cell equivalents) prepared as described in Materials and Methods.
Autosomal recessive CGD with absent cytochrome b absorbance spectrum (A−) is a rare form of CGD and has been reported in only eight patients.11,14,16,17 The mutation responsible for this type of CGD, while not yet identified, must somehow interfere with the expression of cytochrome b. Figure 2 shows Western blots of neutrophils from four patients with A− CGD in a format that is similar to that in Fig 1. In the upper panel, normal levels of the 91kD subunit are seen both in the purified cytochrome b (lane 1) and in the two controls (lanes 2 and 3). Lanes 4 through 7 are from one male and three female A− CGD patients. The 91kD subunit is undetectable in all of these patients. The lower panel shows that a similar pattern is observed for the 22kD subunit. Again, neutrophils from the four A− patients in lanes 4 through 7 are devoid of the 22kD subunit.

**DISCUSSION**

It has been clearly established that genetic absence of the 91kD subunit of human neutrophil cytochrome b results in the X− form of CGD21 and that this condition is also characterized by the absence of the 22kD subunit.8,19 In the present study, we have extended this finding to three additional X− patients. Furthermore, our results show that in A− CGD, both cytochrome b subunits are present at normal levels—a result consistent with the fact that the cytochrome b spectral content is normal and that the defect in this form of the disease involves the cytosolic, not the membrane, components of the oxidase.5,7,18

A remaining puzzling feature of CGD is that two genetically distinct forms of the disease, X− and A−, are characterized by the same defect: the spectral absence of the cytochrome b component of the respiratory burst oxidase. A possible molecular basis for this heterogeneity was suggested by the purification of cytochrome b which demonstrated that it is a heterodimeric protein consisting of subunits of 91kD and 22kD.8,18 Because of the dual subunit structure of this heme protein, we hypothesized that each of the two cytochrome-negative forms of CGD might be due to a defect in one or the other subunits of the cytochrome in a complementary fashion. The data presented in this report do not, however, support this hypothesis. Both subunits of the cytochrome b heterodimer are absent in X− CGD despite the fact that the genetic lesion affects only the 91kD subunit. Moreover, in A− CGD, which is genetically distinct from the X− form, both cytochrome b subunits are also missing. One interpretation of these results is that the stable expression of each of the cytochrome b subunits requires the presence of the other.

In support of this interpretation is the finding that the mRNA for the 22kD, but not the 91kD, subunit is present in a number of nonmyeloid cell type cells including cultured human endothelial cells.22 These cells do not have measurable levels of the 22kD protein despite the presence of the message. Thus, their lack of the 91kD message (and therefore protein) may have precluded the expression of the 22kD protein in a manner analogous to what we have observed in the genetically defective X− CGD neutrophils.

The converse situation may pertain to the A− CGD patients. Failure to transcribe or translate the 22kD gene would result in the absence of the 91kD protein even in the presence of a normal 91kD mRNA. Alternatively, a more complex mechanism of translational or transcriptional control could be involved. The simplest interpretation, however, is that in both X− and A− CGD, the stable expression of each of the cytochrome b subunit proteins requires the presence of the other. Based on these findings, we predict that the defect in A− CGD will be found to involve the gene coding for the 22kD subunit.

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