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33. We thank J. A. Adler for providing the *E. coli* strain AW551 and G.-Y. Lu for purifying and crystallizing the mutant protein. Supported by the Howard Hughes Medical Institute and grants from the National Institutes of Health and the Welch Foundation. Coordinates have been offered for deposit with the Brookhaven Data Bank.

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Two Cytosolic Neutrophil Oxidase Components Absent in Autosomal Chronic Granulomatous Disease

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Neutrophils kill microorganisms with oxygen radicals generated by an oxidase that uses the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as substrate. This system requires both membrane and cytosolic components and is defective in patients with chronic granulomatous disease. A cytosolic complex capable of activating latent membrane oxidase was eluted from guanosine triphosphate–agarose and was used to raise polyclonal antiserum that recognized 47- and 67-kilodalton proteins. These proteins were restricted to the cytosol of myeloid cells. Both proteins were associated with NADPH oxidase–activating capacity when neutrophil cytosol was purified on nucleotide affinity matrices or molecular sizing columns. Neutrophils from patients with two different forms of autosomal chronic granulomatous disease lacked either the 47- or 67-kilodalton protein.

THE MICROBICIDAL ACTIVITY OF POLYmorphonuclear neutrophils (PMNs) depends on a burst of nonmitochondrial oxidative metabolism that converts molecular oxygen to superoxide anion and other toxic oxygen derivatives. The biochemical basis for this respiratory burst is the stimulus-dependent activation of an NADPH oxidase (*1*). This enzyme system consists of (i) membrane-associated catalytic components that include cytochrome b_{558} and function as an electron transport chain (*2–4*) as well as (ii) soluble cytosolic components that appear to be involved in oxidase activation (*4, 5*). Chronic granulomatous disease (CGD), a clinical syndrome of severe and recurrent infections, is characterized biochemically by the absence of the respiratory burst (*1, 6*). The X-linked form is associated in most cases with an absence of cytochrome b and an inherited defect in its 91-kD β subunit (*1, 6, 7*). In contrast, autosomal CGD, constituting about one-third of the cases, is generally characterized by normal cytochrome b but an absence of the essential cytosolic factor activity (*8–10*).

Several laboratories have used a system of subcellular components to reconstitute a functioning oxidase system in vitro (*4, 5*). In this cell-free system, the latent oxidase is activated and NADPH-dependent superoxide formation expressed when PMN plasma membrane or specific granule membrane fractions are combined with cytosol, Mg^{2+}, and either arachidonic acid or SDS. Activation of the reconstituted system is augmented by guanosine triphosphate (GTP) analogs or fluoride and inhibited by guanosine diphosphate (GDP) analogs, suggesting involvement of a GTP-binding protein (*11*). In addition, we have recently noted the presence of a substrate for pertussis toxin–catalyzed adenosine diphosphate (ADP)–ribosylation in PMN cytosol (*12*). On the basis of these observations, we reasoned that the cytosolic factor important in activation might be a GTP-binding protein and we attempted to purify this factor by GTP-agarose affinity chromatography. Fractions were assessed for superoxide-generating activity in the presence of PMN membrane fractions, arachidonic acid, and NADPH (Fig. 1). All of the activity in cytosol was bound to the column and was eluted with a

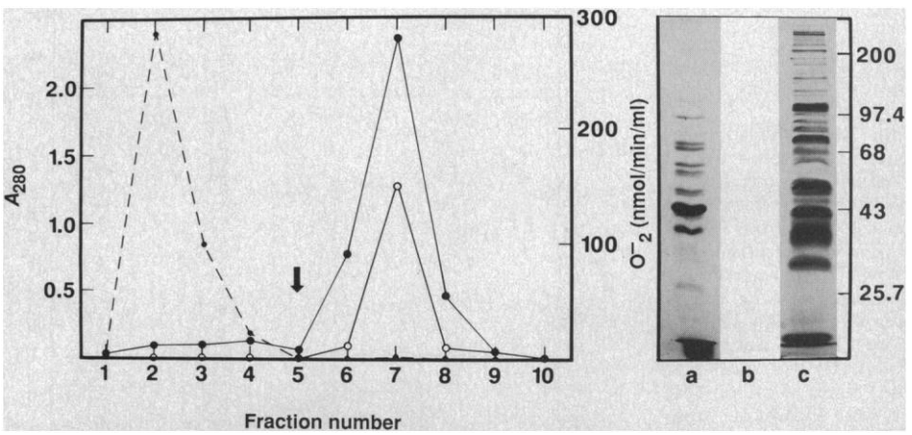

Fig. 1. Partial purification of PMN cytosol factor on a GTP-agarose affinity column. Human PMNs (*18*) were purified, disrupted by nitrogen cavitation, and fractionated on Percoll gradients (*4*). (**Left**) Cytosol from 2×10^8 cells was loaded on a column containing 1 ml of GTP-agarose (*19*). The column was then washed with 5 ml of buffer followed by elution with 5 ml of buffer (arrow) (*19*). Fractions of 1 ml were collected and assessed for protein (– – –) and NADPH oxidase activation (*4, 20*) (———) in the presence (●) or absence (○) of 5 μ*M* GTP-γ-S (Boehringer Mannheim). (**Right**) SDS-PAGE on 9% slab gels (*21*). Shown are Coomassie blue stains of (a) PMN cytosol (5×10^6 cell equivalents) and (b) the active fraction from the GTP-agarose column (~40×10^6 cell equivalents) and (c) a silver stain of the same quantity of the active column fraction. The locations of molecular size standards are shown in kilodaltons.

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high salt buffer containing either GTP or ATP. Eluate activity was enhanced by addition of guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) and coincided with the major peak of GTP-binding capacity (*13*). Most of the cytosolic protein did not bind to the column and thus the eluted material was of high specific activity (30- to 100-fold purification) although many discrete proteins were present (Fig. 1, lane c). Recovery (60 to 100%) was much higher than we could achieve with ion exchange or molecular sieve column chromatography. We hypothesized that the cytosol factor exists as a functional complex that binds to GTP-agarose but is separated into individual components on nonaffinity chromatography media.

We next raised a polyclonal rabbit antiserum (B-1) to the partially purified cytosol factor and used it to screen immunoblots of proteins present in PMN fractions (Fig. 2). The antiserum recognized two predominant proteins in PMN cytosol, migrating in SDS–polyacrylamide gel electrophoresis (PAGE) with molecular masses of 47 and 67 kD. This selectivity of the antiserum suggests that, relative to other components of partially purified cytosol, the 47- and 67-kD proteins are particularly immunogenic. Less intense bands, particularly at 30 and 69 kD, were sometimes detected. There were no immunoreactive proteins in azurophilic granules or the plasma membrane, although there was a large molecule (~84 kD) in fractions enriched for specific granules. Gels run under reducing and nonreducing conditions were identical, suggesting there was no sulfhydryl cross-linking of the detected species. A variety of cells of myeloid and nonmyeloid origin were also screened. Prominent bands of 47 and 67 kD were seen in monocytes (and the monocytoid cell line U937) and differentiated HL-60 cells. Both of these types of cells exhibit a respiratory burst and have oxidase-activating activity in their cytosol (*1, 5, 14*). In contrast, undifferentiated HL-60 cells, lymphocytes, platelets, erythrocytes, endothelial cells, Madin-Darby canine kidney (MDCK) cells, and fibroblasts, all cells without a respiratory burst, lacked the two proteins found in myeloid cells. A diffuse band at 65 to 70 kD in lymphocytes, fibroblasts, and endothelial cells and a prominent 30-kD erythrocyte band are of uncertain significance at this time.

We next compared NADPH oxidase-activating ability with immunoblots of fractions obtained from columns of GTP-agarose, 2′,5′-ADP–agarose (*15*), and a molecular sizing high-performance liquid chromatography (HPLC) column (Fig. 3). With both affinity columns there was a single narrow peak in eluate fractions that contained nearly all of the detectable oxidase activity as well as the 47- and 67-kD proteins. The coelution of both proteins from GTP-agarose and 2′,5′-ADP–agarose suggests that they bind to the columns as a noncovalent complex or, alternatively, that each individual protein has an affinity for the nucleotides. In the sizing column, the 67-kD protein eluted slightly before the 47-kD species as expected, but there was a significant area of overlap and it was these overlapping fractions that had the only detectable superoxide-generating activity. Thus, in each case, the 47- and 67-kD proteins detected by immunoblotting cosegregated with superoxide-supporting activity, consistent with the hypothesis that these proteins represent the active species in cytosol.

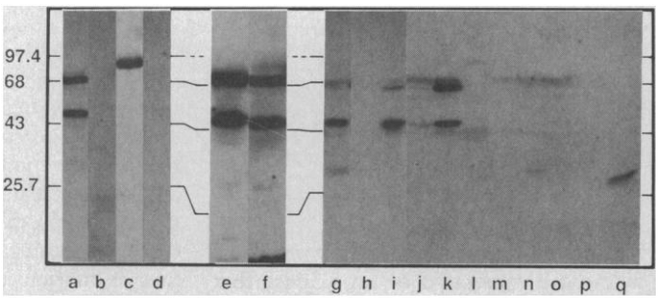

Fig. 2. Immunoblots of cells and fractions with B-1 antiserum. Samples were separated by electrophoresis as in Fig. 1 and proteins were electroblotted to nitrocellulose sheets that were exposed sequentially to blocking reagent, a 1:200 dilution of B-1 antiserum (*22*) and ^{125}I-labeled protein A (*23*). Autoradiography was done by exposing Kodak XAR film to the blots at −80°C for 12 to 48 hours. (Lanes a to d) Subcellular fractions from human PMNs (*4*) were those enriched for (lane a) cytosol, (lane b) azurophilic granules, (lane c) specific granules, and (lane d) plasma membranes. Each lane contained 5×10^6 cell equivalents (except lane c which had 10^7). (Lanes e and f) PMN cytosol was separated by electrophoresis under (lane e) reducing or (lane f) nonreducing conditions. (Lanes g to q) Whole cells were lysed in 1% Triton X-100 (in 100 m*M* tris-HCl, *p*H 7.4, plus protease inhibitors). Fractions of 5×10^6 cell equivalents of each cell type (except for 5×10^7 platelets) were separated by electrophoresis and blotted. (Lane g) PMNs, (lane h) uninduced HL-60 cells, (lane i) HL-60 cells induced to differentiate by growth in 1.25% dimethyl sulfoxide (DMSO) for 6 days, (lane j) U937 cells, human blood (lane k) monocytes, (lane l) platelets, and (lane m) lymphocytes, (lane n) human foreskin fibroblasts, (lane o) human umbilical vein endothelial cells, (lane p) MDCK cells, and (lane q) human erythrocytes. When blots of PMN cytosol were probed with preimmune rabbit serum no immunoreactive bands were detected.

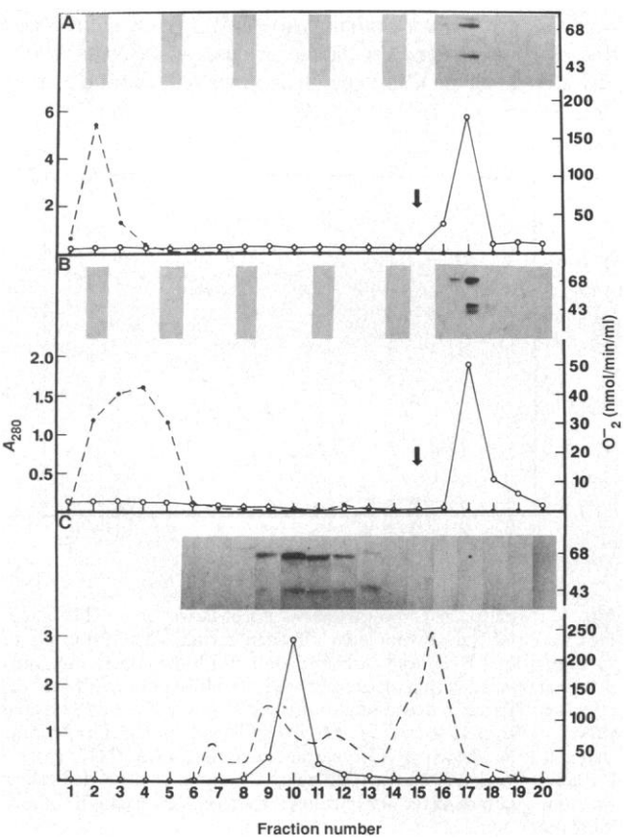

Fig. 3. Coisolation of NADPH oxidase-activating capacity with the 47- and 67-kD immunoreactive proteins. Cytosol from 3×10^8 PMNs was fractionated on (**A**) GTP-agarose [as in Fig. 1 except that the column was washed with 15 ml of buffer before eluting (arrow)], (**B**) 2′,5′-ADP–agarose (Sigma, 6-carbon spacer; wash and elution protocol as for GTP-agarose), and (**C**) HPLC molecular sizing column (*24*). Fractions were assessed for protein (A_{280}, ---), NADPH oxidase activation [Fig. 1 and (*20*)] (———), and proteins reactive with B-1 antiserum in immunoblots (insets, markers of 43 and 68 kD as indicated).

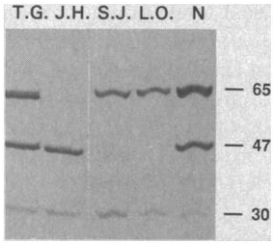

Fig. 4. Immunoblot analysis of PMN cytosols from different genetic forms of CGD. Subjects were: T.G., X-linked cytochrome b–negative CGD; J.H., S.J., and L.O., three autosomal cytochrome b–positive CGDs; and N, normal control. Cytosol fractions of patient cells were prepared from PMN sonicates by sequential centrifugation (12,000*g*, then 130,000*g*) and 10^6 cell equivalents of the final supernatant were loaded in each lane. Conditions were as in Fig. 2 except that proteins immunoreactive with B-1 antiserum were detected with peroxidase-conjugated goat antibody to rabbit immunoglobulin and 4-chloro-1-naphthol as substrate. The locations of molecular size standards are shown in kilodaltons.

On the basis of cytosol-mixing experiments, Nunoi *et al.* (*10*) have demonstrated two complementary defects in cytosol factors in different autosomal CGD patients. To test our hypothesis that the proteins recognized by B-1 antiserum were the active cytosolic factors, cytosols from patients with various forms of CGD were analyzed for the presence of immunoreactive proteins (Fig. 4). Cytosol from a representative individual (T.G.) with X-linked CGD (cytochrome b–negative) contained the two proteins, consistent with the primary defect in the patient's PMNs being the absence of cytochrome b in the presence of normal cytosol activity. In contrast, the cytosols from two unrelated individuals with autosomal CGD (S.J. and L.O.) lacked the 47-kD protein. One patient, J.H., an individual with autosomal CGD but with a different defect functionally complementary to that of the other autosomal patients (*10*), had the 47-kD protein but lacked the 67-kD protein.

We conclude that two cytosolic proteins of 47 and 67 kD are essential for superoxide production by PMNs. That both of these cytosolic factors are critical components of the PMN respiratory burst machinery is validated by their selective absence from neutrophils of patients with autosomal CGD. Our immunochemical demonstration of two distinct forms of autosomal CGD deficient in either the 47- or 67-kD cytosolic oxidase components is supported by the functional studies of Nunoi and colleagues (*10*).

The structure and function of the two proteins we have described are not yet known. The 47-kD species may be the PMN phosphoprotein that undergoes stimulus-dependent phosphorylation in normal but not CGD cells (*16*) and in cell-free systems reconstituted with normal but not autosomal CGD cytosol (*9*). As for the 67-kD protein, some purified NADPH oxidase preparations contain a catalytic component of about this size (*3*), and substrate affinity-labeling techniques have implicated a 65- to 66-kD oxidase-associated PMN protein as the NADPH-binding component (*17*). Moreover, the 2′,5′-ADP affinity of macrophage cytosol factor has been attributed to NADPH binding site specificity (*15*). Whether the 47- and 67-kD factors operate independently or function together, how they interact with or possibly bind to the membrane-associated oxidase components during activation, what covalent modifications (such as phosphorylation) occur during activation, and whether their affinity for GTP has functional significance will need to be determined. Antibodies to these proteins will facilitate their purification, structural characterization, and molecular cloning.

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18. Bleeding of human subjects after obtaining informed consent and generation of antiserum in rabbits were carried out under protocols approved by the appropriate institutional committees.
19. GTP-agarose (6-carbon spacer, Pharmacia) columns were run as follows: at 4°C the sample was loaded, column incubated for 1 hour and wash buffer [100 m*M* KCl, 3 m*M* NaCl, 3.5 m*M* $MgCl_2$, 10 m*M* 1,4-piperazinediethanesulfonic acid (Pipes), *p*H 7.3] was applied; elution buffer [wash buffer plus 150 m*M* NaCl and 1 m*M* ATP $(Na)_2$] was then applied at 25°C. ATP could be replaced by an equal concentration of GTP.
20. Rates of superoxide formation were determined at 37°C in a dual beam spectrophotometer with 0.5-ml cuvettes containing 50 to 100 μl of column fraction to be tested plus 0.1 m*M* cytochrome c (Fe^{3+}), 0.16 m*M* NADPH, 90 μ*M* arachidonic acid, 10^7 cell equivalents each of PMN plasma membrane and specific granule fractions (*4*), PiCM buffer (138 m*M* NaCl, 2.7 m*M* KCl, 0.6 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 10 m*M* phosphate buffer, *p*H 7.4), and in the reference cuvette only, 25 μg superoxide dismutase. Cytochrome c reduction (superoxide formation) was calculated from the rate of increase in the absorbance at 550 nm (A_{550}) with the use of a specific absorbance of 21.1/m*M*/cm (*4*).
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22. A 2.5-kg New Zealand white rabbit was immunized with four subcutaneous injections (100 μg of protein) at 2-week intervals. The immunogen was the peak activity fraction from the GTP-agarose column (Fig. 1) diluted 1:1 with complete or incomplete Freund's adjuvant for the first or second injection, respectively, and undiluted for subsequent injections.
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24. HPLC was done at 4°C with a Beckman TSK-3000 SW column, a buffer of 40 m*M* NaCl, 2 m*M* $MgCl_2$, and 10 m*M* phosphate at *p*H 7.4, and fractions of 750 μl each. Molecular mass standards blue dextran (V_0), transferrin (80 kD), ovalbumin (45 kD), and cytochrome c (12.5 kD) eluted in fractions 6, 9, 12, and 20, respectively.

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