Phosphorylation of the Oxidase-Related 48K Phosphoprotein Family in the Unusual Autosomal Cytochrome-Negative and X-Linked Cytochrome-Positive Types of Chronic Granulomatous Disease


Activation of 32P-loaded neutrophils with phorbol myristate acetate causes the labeling of a family of three 48K proteins that focus near neutral pH. The relationship between these phosphoproteins and the activation of the respiratory burst has been supported by the previous finding that phosphorylation was defective in the two most common types of chronic granulomatous disease (CGD): X-linked cytochrome-negative (X/ -) and autosomal cytochrome-positive (A/+). In this report, these studies have now been extended to the rare A/- and X/+ forms of the disease. In all three patients with A/- CGD examined, the two most acidic 48K proteins failed to undergo enhanced phosphorylation in response to phorbol stimulation, a finding similar to that seen in X/- patients. In contrast, neutrophils from two patients with X/+ CGD appeared to phosphorylate the neutral 48K proteins in a normal fashion. It thus appears that the different phosphorylation patterns seen in chronic granulomatous disease are a reflection of the genetic heterogeneity of this disorder. These findings lend further support to the conclusion that the 48K phosphoprotein family is related to the respiratory burst, although not necessarily in a straightforward manner.

THE RESPIRATORY BURST is a metabolic event characteristic of professional phagocytes in which potent microbicidal agents are generated by the partial reduction of oxygen.1 Chronic granulomatous disease (CGD) is the name given to a group of genetic disorders in which phagocytes are unable to express the respiratory burst. Inheritance of CGD is variable: some types, including the most common, are inherited in an X-linked manner, while others show autosomal recessive transmission.2,3 Regardless of their mode of inheritance, however, all types of CGD are characterized by an abnormally in the respiratory burst oxidase,4 a complex membrane-bound enzyme that catalyzes the production of O2· - from oxygen and NADPH, the key reaction of the respiratory burst. Among the elements of this complex enzyme is a heme protein designated cytochrome b558, that is widely though not universally believed to serve as the oxygen-reducing component of the oxidase.6 The occurrence of this heme protein also varies among different types of CGD: it is present in some types but absent in others.1,7,8-10 It was originally postulated that in CGD the presence or absence of cytochrome b558 correlated with the mode of inheritance.7 More recent studies, however, have failed to confirm this correlation, and types of CGD have been identified that correspond to all four possible combinations of genetic transmission and heme protein status: X-linked cytochrome-negative (X/-), autosomal-recessive cytochrome-negative (A/-), X/+ , and A/+ .1,7,11

The respiratory burst oxidase is dormant in resting phagocytes and cannot be activated by T-cell-derived cytokines. The relationship between these neutrophils and the cells they activate is unknown. The stimulation of neutrophils by T-cell-derived cytokines may be described in a straightforward manner. In the present study, we used a method of activation of the oxidase that involves the stimulation of T-cell-derived cytokines. The stimulation of neutrophils is accomplished by the addition of T-cell-derived cytokines to the culture medium. The stimulation of neutrophils is accomplished by the addition of T-cell-derived cytokines to the culture medium. The stimulation of neutrophils is accomplished by the addition of T-cell-derived cytokines to the culture medium.

MATERIALS AND METHODS

Materials. 32P-labeled phosphoric acid (carrier free in 0.02 mol/L HCl) was obtained from ICN Immunologicals (Chicago). Nitro blue tetrazolium (NBT), Triton X-100, protease inhibitors (N-ethylmaleimide, iodoacetic acid, leupeptin, pepstatin, and diisopropyl fluorophosphate), PMA, N-formylmethionylleucylphenylalanine (FMLP), prestained molecular weight markers, and Nonidet P-40 were purchased from Sigma Chemical Co, St Louis. Benazmide was obtained from Aldrich Chemicals, Milwaukee. Macrodex and Ficoll-Paque were obtained from Pharmacia Laboratories, Piscataway, NJ. Sodium dodecyl sulfate, acrylamide, N,N'-methylenebisacrylamide, Comassie brilliant blue R-250, mercaptoethanol, and agar were obtained from Bio-Rad Laboratories, Richmond, CA; pl markers from Calbiochem-Behring Corp, La Jolla, CA; and ultrapure urea from Schwarz/Mann Biotech, Cleveland. Other materials were the best quality commercially available and were used without further purification.

Preparation, 32P loading, activation, and gel electrophoretic analysis of human neutrophils. These procedures were carried out exactly as described by Okamura et al14 except that for some of the experiments the NaF, N-ethylmaleimide, iodoacetic acid, (NH4)2- MoO4, and benzamidine were omitted from the inhibitor buffer in which the incubations were terminated. This modification, which

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Superoxide production and NBT reduction by intact neutrophils. Intact cell superoxide (O$_2^-$) production was measured as previously described using the continuous assay for O$_2^-$-dependent cytochrome c reduction.$^{17}$ PMA, FMLP, and arachidonic acid were used as stimuli. For most of the patients, NBT reduction was determined by using the same reaction mixture except that NBT (1 mg/mL) was substituted for cytochrome c. After five minutes of stimulation with PMA (2 μg/mL), the reaction mixture was placed on ice. A sample of the mixture was placed in a hemocytometer, and the percent of cells containing the purple formazan was determined microscopically. For the patient with X/+ CGD and his family members, however, the NBT test was performed as described by Ochs and Igo.$^{18}$

Neutrophil glucose-6-phosphate dehydrogenase activity. Glucose-6-phosphate dehydrogenase activity was measured in digitonin extracts of purified neutrophils by the method of Lühr and Waller.$^{19}$ Enzyme activity was determined at two concentrations of cell extract to ensure linearity with respect to enzyme concentration. Results are expressed as means of the two determinations, which agreed within 5%.

Cytochrome b$_{558}$. Neutrophils were disrupted by nitrogen cavitation using a modification$^{11}$ of the method described by Borregaard et al.$^{20}$ The cavitate was centrifuged at 500 g for ten minutes at 4°C to remove nuclei and the few remaining undisrupted cells (<5%). The level of cytochrome b$_{558}$ was then measured in this postnuclear supernatant by means of a dithionite reduced-oxidized difference spectrum as previously described.$^{11}$

RESULTS

Phosphorylation was studied in neutrophils from three patients with A/− CGD. Two of the three were female, and in all three the family histories and the results of laboratory investigations were consistent with the diagnosis of autosomal inherited CGD (Table 1). Neutrophil cytochrome b$_{558}$ was not detected in any of these patients.

R.C. and D.C., two brothers with X/+ CGD, are white males aged 33 and 31, respectively. Each has had a history of recurrent bacterial infections since childhood, and in each, CGD was diagnosed on the basis of clinical features and the failure of their neutrophils to manufacture O$_2^-$ in response to PMA$^{18,25-28}$ (O$_2^-$ production, 0.2 [R.C.] and 0.6 [D.C.] nmol/min/10$^7$ patient cells as compared with control values of 107.3 and 122.5 nmol/min/10$^7$ normal cells, respectively). Their mother is a carrier, as are two of R.C.’s female children (Fig 2). The glucose-6-phosphate dehydrogenase activity in R.C.’s granulocytes was normal (222 nmol NADPH/min/10$^7$ cells, 9.9 pmol/10$^7$ cells). The cytochrome b$_{558}$ level in R.C.’s neutrophils was 56.2 pmol/10$^7$ cells and in D.C.’s neutrophils, 60.7 pmol/10$^7$ cells, both values well within the normal range of 42.8 ± 9.9 pmol/10$^7$ cells.

The results of the phosphorylation studies in the A/− patients are shown in Fig 3. As reported elsewhere,$^{13,14}$

Table 1. Clinical and Laboratory Features of the Three Patients With A/− CGD Examined in This Study

<table>
<thead>
<tr>
<th>Patient/Sex</th>
<th>Family History</th>
<th>NBT Test (Percentage Positive)</th>
<th>O$_2^-$ Formation (nmol/min/10$^7$ PMN)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.N./F</td>
<td>Parents first cousins. NBT test result normal in both parents, all 3 siblings (2 M, 1 F) and 1 child (F)</td>
<td>0</td>
<td>0</td>
<td>11, 21, 22</td>
</tr>
<tr>
<td>G.S./M</td>
<td>NBT test result normal in 3 grandparents, both parents and 5 siblings (4 F, 1 M). One female sibling died of CGD complications</td>
<td>0</td>
<td>0.6</td>
<td>11, 23*, 24*</td>
</tr>
<tr>
<td>A.V./F</td>
<td>Normal NBT test result in both parents and all 3 siblings (M)</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

*The female sibling of patient G.S. is described as case 2 in these reports.
stimulation by PMA of $^{32}$P-loaded neutrophils from normal subjects resulted in the uptake of label by a group of three closely related 48K proteins that focused at pI 6.8, 7.3, and 7.8 (the neutral 48K proteins, individually designated as pp48/6.8, pp48/7.3, and pp48/7.8; see Fig 1, arrows, right to left, respectively). Of these three, only pp48/7.8 acquired significant amounts of additional label in cells from patients with $A/-$ CGD. This finding is similar to observations made with cells from patients with the X/− type of CGD (see Fig 4, right panel).13,14

Results obtained with neutrophils from the X/+ patients are shown in Fig 4. Somewhat surprisingly, their neutrophils appeared to phosphorylate the neutral 48K proteins in a completely normal fashion. With D.C.'s cells, this finding was confirmed by direct measurement of the quantities of $^{32}$P in these proteins (Table 2 and Fig 5). These observations stand in contrast to the results of previous studies in several patients with $A/-$ CGD whose neutrophils failed in every case to phosphorylate any of the proteins of the neutral 48K group.13,15

### DISCUSSION

Because so few patients with X-linked cytochrome-positive CGD have been reported, there has been some question about the existence of this type of the disease. Our studies on R.C. and D.C., however, indicate that X/+ CGD is a real entity. X-linked transmission of CGD in this family is well supported by the patient's pedigree, which in addition to two affected brothers includes three first-degree female relatives whose neutrophils show chimerism for respiratory burst activity. Spectroscopy has demonstrated normal amounts of cytochrome $b_{558}$ in neutrophils from both these patients. Moreover, R.C. and D.C. are unique in that among patients with CGD, theirs are the only neutrophils studied to date that have shown a normal pattern of phosphorylation of the neutral 48K proteins. X/+ CGD thus appears to be a separate type of the disease, with features that clearly distinguish it from the other types hitherto described.

Studies to date have revealed a complex pattern of phosphorylation defects in patients with various forms of CGD

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**Table 2. $^{32}$P Content of the Neutral 48K Phosphoproteins in $^{32}$P-Loaded Activated Neutrophils From a Normal Subject and a Patient With X/+ CGD**

<table>
<thead>
<tr>
<th>Phosphoprotein</th>
<th>Normal (cpm)</th>
<th>Normal (%)</th>
<th>X/+ CGD (cpm)</th>
<th>X/+ CGD (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp48/7.8</td>
<td>31</td>
<td>31</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>pp48/7.3</td>
<td>46</td>
<td>46</td>
<td>41</td>
<td>44</td>
</tr>
<tr>
<td>pp48/6.8</td>
<td>77</td>
<td>77</td>
<td>66</td>
<td>72</td>
</tr>
<tr>
<td>pp53/8.0</td>
<td>100</td>
<td>(100)</td>
<td>92</td>
<td>(100)</td>
</tr>
<tr>
<td>Activated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pp48/7.8</td>
<td>266</td>
<td>228</td>
<td>205</td>
<td>187</td>
</tr>
<tr>
<td>pp48/7.3</td>
<td>349</td>
<td>299</td>
<td>299</td>
<td>272</td>
</tr>
<tr>
<td>pp48/6.8</td>
<td>606</td>
<td>519</td>
<td>364</td>
<td>332</td>
</tr>
<tr>
<td>pp53/8.0</td>
<td>117</td>
<td>(100)</td>
<td>110</td>
<td>(100)</td>
</tr>
</tbody>
</table>

The experiment was carried out as described in Materials and Methods. The locations of the excised phosphoproteins as determined by autoradiography are shown in Fig 5. The phosphoprotein pp53/8.0 was used to normalize the data since it did not undergo enhanced phosphorylation following stimulation.

*Expressed as percentage of counts per minute in pp53/8.0.
Resting Activated

NI

xI+

(R.C.)

Fig 4. Phosphorylation of the 48K protein group in neutrophils from two patients with X/+ CGD. Experiments were carried out as described in the text. (Left) Protein phosphorylation by neutrophils from R.C. and a concurrently studied normal control. In these experiments, the incubations were terminated with the original inhibitor buffer. (Right) Protein phosphorylation by concurrently studied neutrophils from D.C., a normal control, and a patient with X/- CGD. In these experiments, the incubations were terminated with the modified inhibitor buffer from which the sulfhydryl reagents and phosphatase inhibitors were eliminated (see Materials and Methods). In each panel, the location of the 48K phosphoprotein group is indicated by the arrow. Phosphorylation of the 48K protein group by neutrophils from the patients with X/+ CGD was indistinguishable from normal.

Fig 5. The locations of the phosphoproteins selected for liquid scintillation counting (see Table 2). This figure shows a larger section of the autoradiogram obtained with 32P-loaded, phorbol-activated X/+ neutrophils (patient D.C.) and presented in Fig 4. The 48K phosphoprotein group is indicated by the arrow, while pp53/8.0, a phosphoprotein that appears to be labeled equally in resting and activated neutrophils and was used as a normalizing standard, is indicated by the arrowhead.

3. Many observations suggest that the enzyme most likely to catalyze the phosphorylation of the 48K protein family in activated neutrophils is protein kinase C. Treatment

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>pp48/7.8</th>
<th>pp48/7.3</th>
<th>pp48/8.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>CGD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X/-</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A/-</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>X/+</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>A/+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: +, phosphorylated; 0, not phosphorylated; ±, inconsistently phosphorylated.
of neutrophils with agents that activate this kinase induce the phosphorylation of these proteins in whole cells and cytoplasts, and protein kinase C added directly to neutrophil cytoplasmic lysates also phosphorylates these proteins.

4. Although alternative interpretations are possible, the observation that 48K protein phosphorylation fails altogether in A/+ CGD together with the finding that the molecular defect in this form of CGD involves the cytosol and not the membranes is most simply explained by postulating that the unphosphorylated parent of the 48K phosphoprotein family normally resides in the cytosol and that in A/+ CGD this progenitor polypeptide is defective or missing.

Taken together, these results suggest that the phosphorylation of the neutral 48K protein family takes place in two successive steps occurring in different locations in the neutrophil. The first step is the production of pp48/7.8 by the protein kinase C-dependent phosphorylation of the progenitor polypeptide, a reaction (or series of reactions) that can take place in the cytosol. The newly formed pp48/7.8 then binds to the membrane at a specific site created by cytochrome b$_{558}$ and is there further phosphorylated to form pp48/6.8 and pp48/7.3, again by protein kinase C, but this time only when the protein kinase is membrane bound. In the absence of cytochrome b$_{558}$, pp48/7.8 is generated during neutrophil activation but cannot be further phosphorylated because its membrane binding site is missing.

Though this interpretation explains the protein phosphorylation patterns seen in the various forms of CGD, it says nothing about the functional relationship between the phosphorylation of these proteins and the operation of the respiratory burst oxidase. This relationship, perhaps the most important feature of the neutral 48K phosphoprotein family, remains completely obscure. It is clear that much further work will be needed to elucidate the role of these enigmatic phosphoproteins in the respiratory burst.

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Phosphorylation of the oxidase-related 48K phosphoprotein family in the unusual autosomal cytochrome-negative and X-linked cytochrome-positive types of chronic granulomatous disease

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