Characterization of Two Monoclonal Antibodies Against Cytochrome b\textsubscript{558} of Human Neutrophils

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Monoclonal antibodies (MoAbs) were raised against cytochrome b\textsubscript{558}, a membrane-bound component of the NADPH:O\textsubscript{2} oxidoreductase in human neutrophils. This cytochrome consists of a low-molecular-weight (low-mol-wt) subunit of 22 to 23 Kd, probably encoded by an autosomal gene, and a high-mol-wt subunit of 75 to 90 Kd, encoded on the X-chromosome. MoAb 449 reacts with the low-mol-wt subunit and MoAb 48 with the high-mol-wt subunit on Western blots of purified cytochrome b\textsubscript{558} and on blots of whole neutrophil extracts. In extracts of neutrophils from patients with chronic granulomatous disease (CGD) in which cytochrome b\textsubscript{558} is not detectable by spectrophotometric methods, the low-mol-wt subunit is present, albeit in a much smaller amount. The high-mol-wt subunit is not detected by MoAb 48 in neutrophils of patients with X-linked CGD and in neutrophils of patients with the autosomal, cytochrome-b\textsubscript{558}-negative form of the disease. These results can be explained by a marked instability of these subunits when the synthesis of either of the two is disturbed. In differentiated HL-60 cells, the high-mol-wt subunit appears to be present in a different form. Cloning of the low-mol-wt subunit with the help of MoAb 449 suggests the presence of a heme-binding site on this subunit. By comparison of the binding characteristics of MoAb 449 to intact and permeabilized neutrophils with those of MoAb 7D5, recently isolated by Nakamura et al (Blood 69:1404, 1987), the low-mol-wt subunit was established as a transmembrane protein.

Cytchrome b\textsubscript{558} is a membrane-bound component of the NADPH:O\textsubscript{2} oxidoreductase present in phagocytic cells.\textsuperscript{1} The NADPH:O\textsubscript{2} oxidoreductase is dormant in resting cells but is rapidly activated after addition of opsonized particles or soluble stimuli, such as phorbol esters or chemotactic peptides. After activation, the enzyme produces superoxide (O\textsubscript{2}\textsuperscript{-}), a process generally known as the "respiratory burst" and essential for the killing of ingested bacteria. Inherited defects in the activity of the NADPH:O\textsubscript{2} oxidoreductase\textsuperscript{2} are the reason for recurrent infections observed in patients with chronic granulomatous disease (CGD). In two genetically different forms of CGD, the light absorbance of the 75- to 90-Kd glycoprotein and a 22- to 23-Kd protein. On the basis of amino-acid sequence identity\textsuperscript{3} and Western-blot analysis with polyclonal antisera,\textsuperscript{9} it was concluded that the 75- to 90-Kd glycoprotein represents the product of the gene abnormal in X-linked CGD. The underlying defect in the autosomal form of cytochrome b\textsubscript{558}-negative CGD might be due to a defect in the gene encoding the 22- to 23-Kd subunit.\textsuperscript{14}

To facilitate study of the structure of cytochrome b\textsubscript{558} and analysis of the defects observed in CGD, we decided to raise monoclonal antibodies (MoAbs) against the cytochrome b\textsubscript{558} complex of human neutrophils. Until now, only one MoAb (designated 7D5) has been described.\textsuperscript{15} We now report the isolation and characterization of two MoAbs specific for cytochrome b\textsubscript{558}. MoAb 449 binds to cytochrome b\textsubscript{558} under a variety of experimental conditions; it recognizes the low-mol-wt subunit of cytochrome b\textsubscript{558}. MoAb 48 binds only to detergent-solubilized cytochrome b\textsubscript{558} and recognizes the high-mol-wt subunit, as indicated by Western-blot analysis.

MATERIALS AND METHODS

Purification of cytochrome b\textsubscript{558}. Buffy coats were prepared from 24 to 36 U blood (500 mL) that had been stored overnight at room temperature. The buffy coats were diluted twofold with phosphate-buffered saline (PBS) containing 0.4% (wt/vol) trisodium citrate and were pooled. This suspension was layered in 35-mL portions onto a 15-mL isotonic Percoll solution with a specific gravity of 1.074 g/mL at 20°C, and was centrifuged for 15 minutes at 1,000 g at 20°C. The neutrophils and most of the erythrocytes were recovered from below the Percoll layer. The erythrocytes were subsequently lysed at 4°C with an isotonic NH\textsubscript{4}Cl-KHCO\textsubscript{3}-EDTA solution as described previously.\textsuperscript{14} The final preparation of neutrophils contained <5% lymphocytes and thrombocytes.

The following steps were all performed at 4°C. Isolated neutrophils were treated with 2 mmol/L diisopropylfluorophosphate (DFP) for ten minutes. After being washed with PBS, the cells (2 ×
10^6/mL) were homogenized with a Sorvall Omnimixer (16,000 rpm) in a solution containing 0.34 mol/L sucrose, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L EGTA, and 0.1 mol/L potassium phosphate (pH 7.4). A particulate fraction was prepared by centrifugation of the homogenate (40,000 g for 30 minutes), and this fraction was subsequently stored at −70°C until further use. Cytochrome b₅₅₈ was extracted by homogenization of the frozen pellet in 1% (wt/vol) Triton X-114, 1 mmol/L PMSF, 5 mmol/L EGTA, 0.5 mol/L NaCl, and 0.1 mol/L potassium phosphate (pH 7.4). After centrifugation to remove insoluble material, the brownish/green supernatant was subjected to a Triton X-114 phase separation by incubation for 20 minutes at 25°C. The Triton X-114 phase, containing all the cytochrome b₅₅₈, was diluted to the initial volume with a solution containing 20 mmol/L potassium phosphate (pH 6.8), 0.1% (wt/vol) Triton X-100, and 2 mmol/L EGTA. Preswollen carboxymethyl (CM) Sepharose (Pharmacia, Uppsala, Sweden) was added (packed volume 0.5 mL/10 mL detergent-solubilized cytochrome b₅₅₈), and the suspension was stirred overnight. More than 90% of the cytochrome b₅₅₈ was bound to the matrix. The CM-Sepharose was washed three times with a buffer containing 20 mmol/L potassium phosphate (pH 6.8), 0.1% (wt/vol) Triton X-100, 2 mmol/L EGTA, and 50 mmol/L NaCl. The CM-Sepharose was poured in a column, and the cytochrome was eluted in a linear gradient from 50 to 500 mmol/L NaCl in the same buffer. Peak fractions of cytochrome b₅₅₈ (at ~150 mmol/L NaCl) were pooled, concentrated on an Amicon YM10 filter and subsequently layered on top of a column of Sephacryl S-300 (Pharmacia, 1.6 x 100 cm). The column was eluted with a solution containing 0.1% (wt/vol) Triton X-100, 0.2 mol/L NaCl, and 0.1 mol/L potassium phosphate (pH 7.4) at a flow rate of 6 mL/h.

The concentration of cytochrome b₅₅₈ in the various fractions was measured by determination of the reduced-minus-oxidized spectrum on a Hewlett-Packard 8451A diode array spectrophotometer, with 160 mmol/L L-1 cm⁻¹ as the extinction coefficient for the Soret band (A₄₂₈-A₄₄₀). Cytochrome b₅₅₈ in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels was visualized by labeling the protein before electrophoresis by reductive methylation with ¹⁴C-formaldehyde in the presence of cyanoborohydride, according to the method described by Jentoff and Dearborn. Protein content was measured with protein assay reagent containing bicinechonic acid (Pierce Chemical, Rockford, IL) with albumin as a standard.

Production of MoAbs. BALB/c mice were injected intraperitoneally (IP) with 100 μL purified cytochrome b₅₅₈ (2 μmol/L, diluted 1:1 with Freund's complete adjuvant). A second and third injection with 40 μL purified cytochrome b₅₅₈ (without adjuvant) was given IP every 2 weeks. Fourteen days after the last injection, the mice received a booster injection of 40 μL purified cytochrome b₅₅₈ intravenously (IV). Three days later, the spleen cells were fused with SP2/0 Ag 14 cells (Dr G. Köhler, Basel Institute of Immunology, Switzerland) as described previously. Hybrids producing specific antibodies were selected with the enzyme-linked immunosorbent assay (ELISA) described below.

ELISA for detection of antibodies against cytochrome b₅₅₈. For selection of clones producing specific antibodies, an ELISA was developed with the antigen coated on the solid phase. Purified cytochrome b₅₅₈ (200 mmol/L in a solution containing 0.1% (wt/vol) Triton X-100, 0.2 mol/L NaCl, and 0.1 mol/L potassium phosphate, pH 7.4) was diluted 20-fold in coating buffer (140 mmol/L NaCl, 9.2 mmol/L Na₂HPO₄, 1.3 mmol/L NaH₂PO₄, 1.845 mol/L NaCl, 0.5 mol/L L EGTA, 0.05 mol/L L PMSF, 2 mmol/L L HEPES, 110 mmol/L L sucrose, 20 mmol/L L HEPES, 1.0 mol/L L EGTA, 0.5 mol/L L PMSF, and 10 μmol/L L leupeptin, pH 7.0). The postnuclear supernatant was centrifuged over a discontinuous sucrose gradient consisting of 2 mL 50% sucrose and 1 mL 1.076 g/mL at 20°C. The supernatant was subjected to a Triton X-114 phase separation by incubation for 20 minutes at 25°C. The Triton X-114 phase, containing 1% (wt/vol) Triton X-100, 0.2 mol/L NaCl, and 0.1 mol/L potassium phosphate (pH 7.4) at a flow rate of 6 mL/h.

For the anticytochrome b₅₅₈ ELISA, Neutrophils were isolated from blood by centrifugation (50 to 100 mL, collected in heparin and diluted twofold with PBS containing 0.4% trisodium citrate) over a Percoll layer with a density of 1.076 g/mL at 20°C. The supernatant was subjected to a Triton X-114 phase separation by incubation for 20 minutes at 25°C. The Triton X-114 phase, containing 1% (wt/vol) Triton X-100, 0.2 mol/L NaCl, and 0.1 mol/L potassium phosphate (pH 7.4) at a flow rate of 6 mL/h.

The supernatant was subjected to a Triton X-114 phase separation by incubation for 20 minutes at 25°C. The Triton X-114 phase, containing 1% (wt/vol) Triton X-100, 0.2 mol/L NaCl, and 0.1 mol/L potassium phosphate (pH 7.4) at a flow rate of 6 mL/h.

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added to detect antibodies bound to the filter. After 60 minutes, the membrane was washed three times, and color development was initiated by incubation with nitrophenylphosphate and nitroblue tetrazolium for 15 to 30 minutes. The reaction was terminated by rinsing the membrane with aqua dest.

**Purification of MoAbs.** MoAbs were produced as ascites in pristane-primed BALB/c mice. One hour after collection, the ascites were centrifuged (400g for ten minutes) to remove contaminating RBCs and clots. Immunoglobulins were purified from the supernatant on Protein-A Sepharose 4B as indicated by the supplier (Pharmacia). Purified antibodies (protein 0.5 mg/mL) were stored in small aliquots at -70°C with bovine serum albumin (BSA) (5 mg/mL) added.

**Incubation of HL-60 cells.** HL-60 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) with 10% (vol/vol) heat-inactivated fetal calf serum (FCS). Differentiation was induced by exposure of the cells to 1.25% (vol/vol) dimethylsulfoxide (DMSO) for ten days. All other procedures were the same as for freshly isolated neutrophils.

**Binding of antibodies to intact and permeabilized neutrophils.** Freshly isolated neutrophils (2.5 x 10^9/mL) were prepurified for five minutes at 37°C in an incubation medium (pH 7.4) containing 132 mmol/L NaCl, 6 mmol/L KCl, 1.2 mmol/L potassium phosphate, 1 mmol/L MgSO_4, 20 mmol/L HEPES, 1 mmol/L EGTA, and 0.5% human serum albumin (HSA). Subsequently, digitonin was added from a stock solution of 5 mmol/L only. Two minutes after addition of digitonin or DMSO, the cells were centrifuged (400g for five minutes). The cells were washed twice, FITC-labeled goat anti-mouse-Ig (CLB) was added, and incubations were performed with the Protoblot Immunoscreening system (Promega Biotec). Approximately 500,000 plaques of the library (amplified one time) were screened with MoAb 449. Two positive recombinants were identified. The clone with the largest EcoRI insert (680 base pairs, bp) was isolated and amplified by cloning into PUC13. The cDNA was cut with *Hinc*I into two fragments of 400 and 280 bp, respectively. The two fragments were both cloned into M13mp18 and M13mp19, and the single-stranded DNAs were sequenced in both directions with the dideoxynucleotide chain-termination method using the Sequenase kit from USB, Cleveland.

**RESULTS**

Previous isolations of cytochrome *b*_{558} of this laboratory have resulted in a preparation containing a 127-Kd protein as the major constituent, different from the preparations of Segal and Parkos et al. In the present study, we used as antigen a cytochrome *b*_{558} preparation isolated according to a

| Table 1. Purification of Cytochrome *b*_{558} From 70 x 10^9 Neutrophils |
|-------------------------|----------------|----------------|------------------|------------------|
| **Fraction** | **Protein (mg)** | **Total Content (mmol)** | **Specific Content (mmol/mg Protein)** | **Yield (%)** |
| Particulate fraction | 3,600 | 180 | 0.05 | 100 |
| H_2O phase | 3,050 | 0 | 0 | 0 |
| Triton X-114 phase | 550 | 180 | 0.32 | 0 |
| After CM-Sepharose | 44 | 120 | 2.72 | 67 |
| Peak S-300 | 1.9 | 20 | 10.6 | 11 |

Purification of cytochrome *b*_{558} was performed as described in the Materials and Methods section. Values are the mean of two separate isolations.

![Fig 1. Detection of cytochrome *b*_{558} subunits on SDS-polyacrylamide gels. Two fractions, each containing 2 μg protein, were loaded on an SDS-polyacrylamide gel (5% to 15%): (a and c) peak fraction of S-300 column; (b and d) eluate of CM-Sepharose column. Proteins were visualized by reductive methylation with ¹⁴C-formaldehyde (described in the Materials and Methods section) and subsequent autoradiography (left) or by silver staining (right).](www.bloodjournal.org)
scheme essentially different from that used in other studies: homogenization of DFP-treated neutrophils, Triton X-114 phase separation of the particulate fraction, ion-exchange chromatography on carboxymethyl Sephrose and gelpermeation chromatography on Sephacryl S-300 (detailed in the Materials and Methods section). Data on the enrichment of cytochrome b$_{558}$ at various steps in this purification are shown in Table 1. In the final preparation, cytochrome b$_{558}$ was purified 200-fold as compared with the starting extract. After SDS-PAGE, a smear at 70 to 90 Kd and a band at 26 Kd were found (Fig 1). In our experience, these bands can only be visualized in a reproducible manner by methylation of the protein mixture with $^{14}$C-formaldehyde with cyanoborohydride added and subsequent autoradiography.

To screen hybridoma supernatants, an ELISA was used with the antigen coated on the wells of the microtiter plates. Efficient coating of cytochrome b$_{558}$ was obtained by lowering the concentration of the detergent Triton X-100 to <0.005% (wt/vol). From two separate fusions with different mouse spleens, 20 positive clones were obtained.

To evaluate the specificity of the positive clones, the following approaches were used. At first, membranes were isolated from disrupted neutrophils on a discontinuous sucrose gradient and coated on the wells of a microtiter plate. Binding of the antibodies present in the hybridoma supernatants was compared with the binding to membranes derived from CGD neutrophils lacking cytochrome b$_{558}$. Only one MoAb (MoAb 449) was positive with control membranes and negative with Xb or A CGD membranes (Fig 2).

In addition, hybridoma supernatants found positive in the anticytochrome b$_{558}$ ELISA were incubated with Western blots prepared from control and CGD neutrophil extracts. With this procedure, two MoAbs were specific for cytochrome b$_{558}$: MoAb 48 and the MoAb 449 described previously. Figure 3A shows that MoAb 48 binds to a smear at ~75 Kd present in control (lane 1) and Ab$^+$ CGD neutrophil extracts (lane 6). This smear is not present in extracts of CGD neutrophils lacking cytochrome b$_{558}$ (Fig 3A, lanes 3 and 4) and coelutes with the heme signal of a cytochrome b$_{558}$.
preparation on a S-300 column (Fig 4A). These results indicate that on a Western blot MoAb 48 is able to bind to the high-mol-wt subunit (or β subunit) of cytochrome b$_{558}$. In addition, MoAb 48 binds weakly to a low-mol-wt protein of 23 Kd present in control and Ab$^+$ CGD neutrophils (Fig 3A) and in purified cytochrome b$_{558}$ (Fig 4A), suggesting crossreactivity with the low-mol-wt subunit of cytochrome b$_{558}$.

In neutrophil extracts from control donors, a 23-Kd protein is recognized by MoAb 449 (Fig 3B, lane 1). This band also coelutes with the heme signal on the S-300 column (Fig 4B). The slightly higher apparent mol wt of the antigen detected in Fig 4B probably results from the different gel system used in this particular experiment. In neutrophil extracts of six X-linked CGD patients, a weak reaction at the 23-Kd level was observed (Fig 3B, lane 3). An even smaller amount of 23-Kda protein was detected by MoAb 449 in extracts of neutrophils from three Ab$^+$ CGD patients (Fig 3B, lane 4). The antibody also consistently reacts with a protein of 43 Kd, which is not detectable in either CGD neutrophil extracts (Fig 3B) or in purified cytochrome b$_{558}$ (results not shown).

Both MoAbs 48 and 449 are IgG$_1$ antibodies and did not react with extracts of human platelets, lymphocytes, and rat hepatocytes (results not shown), indicating the absence of crossreactivity with other cytochromes. We used these antibodies to investigate expression of the two subunits in differentiating HL-60 cells. On differentiation in vitro with DMSO, HL-60 cells express NADPH oxidase activity and, concomitantly, the amount of cytochrome b$_{558}$ present in these cells increases.$^{24,25}$ On Western blots of DMSO-induced HL-60 cells, MoAb 48 binds to two proteins of ~100 and 120 Kd (Fig 5A, lane 2). These bands are also present in undifferentiated HL-60 cells, albeit in a smaller amount (Fig 5A, lane 1). The 75-Kd glycoprotein recognized by MoAb 48 in normal neutrophils appears to be absent from both undifferentiated and DMSO-induced HL-60 cells. In contrast, the low-mol-wt subunit appears to be present in HL-60 cells in the same form as in control neutrophils, as indicated by the binding of MoAb 449 on the blot (Fig 5B). The amount of this subunit is also increased after granulocytic differentiation (Fig 5B, lanes 7 and 8).

Because MoAb 449 is able to bind to native cytochrome b$_{558}$ present in neutrophil membranes (Fig 2), the binding of MoAb 449 to intact neutrophils was investigated in a flow cytometer. After disruption of resting neutrophils, 10% to 30% of the cytochrome b$_{558}$ is recovered in the plasma membrane fraction.$^{26,27}$ This percentage increases about

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**Fig 4.** Western blot of fractions of the Sephacryl S-300 column containing cytochrome b$_{558}$ incubated with MoAb 48 (A) or with MoAb 449 (B). CM-Sepharose eluate was chromatographed on two separate occasions on Sephacryl S-300 as described in the Materials and Methods section. The heme content of each fraction determined from the reduced-minus-oxidized spectrum (top). A sample of each fraction (50 μL) was loaded on a 12% SDS-polyacrylamide gel, electrophoresed, and blotted onto nitrocellulose (described in the Materials and Methods section). Reaction of each fraction with MoAb 48 (A) or MoAb 449 (B) after Western blotting (bottom).
DMSO as described in the Materials and Methods section. The MoAb 449 (B). HL-60 was observed (Fig 6B). Binding of MoAb Bl 3.9 directed of the plasma membrane with digitonin, binding of MoAb stimulated neutrophils (Fig 6C). Only after permeabilization in lanes 3 and 6. cytochrome b sequence is identical to the sequence recently reported by S. Orkin, Boston) resulted in two differentiated HL-60 cells (S. Orkin, Boston) were loaded (lanes 1 and 7), DMSO-treated HL-60 cells (lanes 2 and 8), and human neutrophils (lanes 4 and 5). No samples were loaded in lanes 3 and 6.

Fig 5. Western blot of HL-60 cells incubated with MoAb 48 (A) or MoAb 449 (B). HL-60 cells were differentiated by addition of DMSO as described in the Materials and Methods section. The cytochrome b$_{558}$ content determined spectrophotometrically increased from 0.8 to 2.2 pmol/10$^6$ cells by the DMSO treatment. Extracts of undifferentiated HL-60 cells were loaded (lanes 1 and 7). DMSO-treated HL-60 cells (lanes 2 and 8), and human neutrophils (lanes 4 and 5). No samples were loaded in lanes 3 and 6.

threefold after stimulation of the cells with fMLP in the presence of cytochalasin B.27 However, MoAb 449 did not bind to either resting neutrophils (Fig 6A) or fMLP-stimulated neutrophils (Fig 6C). Only after permeabilization of the plasma membrane with digitonin, binding of MoAb 449 was observed (Fig 6B). Binding of MoAb Bl 3.9 directed against a 90-Kd protein present on the surface of neutrophils and on the interior of specific granules28 (Fig. 6C) was not affected by the permeabilization procedure (results not shown). These results indicate that the epitope recognized by MoAb 449 is on the cytoplasmic surface of plasma membrane and granules.

Finally, MoAb 449 was used to clone the cDNA encoding the low-mol-wt subunit of cytochrome b$_{558}$. Screening with the antibody of a λ-gt11 expression library constructed from differentiated HL-60 cells (S. Orkin, Boston) resulted in two positive clones. The clone with the largest insert (680 bp) was isolated and sequenced. The sequence of the cDNA encoding the epitope recognized by MoAb 449 is shown in Fig 7. This sequence is identical to the sequence recently reported by Parkos et al.29 who used a polyclonal rabbit antiserum against the low-mol-wt subunit of cytochrome b$_{558}$ to isolate the relevant cDNA. The insert isolated with MoAb 449 lacks 175 bp at the 5'-side of the full-length mRNA, which encode the first 50 N-terminal amino acids of the protein. This indicates that the epitope recognized by MoAb 449 does not reside in this part of the protein.

DISCUSSION

Elucidation of the structure and function of cytochrome b$_{558}$ in the NADPH oxidase system in phagocytic cells would be greatly facilitated by the availability of MoAbs against cytochrome b$_{558}$. Despite several attempts to raise MoAbs, only one has been reported.15 Furthermore, in this study only two specific MoAbs were isolated, possibly reflecting the low immunogenicity of the cytochrome b$_{558}$ complex. MoAb 48 can be used to detect the high-mol-wt (or β) subunit of cytochrome b$_{558}$ after Western blotting, but does not bind to native cytochrome b$_{558}$ in the neutrophil membrane. In contrast, MoAb 449 reacts with the low-mol-wt (or α) subunit of cytochrome b$_{558}$ and binds to cytochrome b$_{558}$ under a variety of experimental conditions. This antibody has successfully been used to detect cytochrome b$_{558}$ in human neutrophils by immunoelectron microscopy (L. Ginsel, J. Onderwater, A. J. Verhoeven and D. Roos, unpublished observations).

Our Western blot analysis with neutrophil extracts of different CGD patients and MoAb 48 (Fig 3A) confirms the absence of the β subunit of cytochrome b$_{558}$ in neutrophils from X-linked CGD patients, previously observed by other investigators.10,11 Some of these patients do, however, respond to treatment with interferon-γ with a partial recovery of respiratory burst activity,10,11 indicating that in these
cases the ability to synthesize (part of) the relevant protein is not completely absent. Neither is the a subunit of cytochrome b559 in neutrophil extracts of Xb CGD patients (Fig 3A), probably reflecting the instability of this subunit when a proper complex with the b subunit cannot be formed.

On a Western blot, MoAb 449 detects a small amount of a subunit of cytochrome b559 in neutrophil extracts of Xb CGD patients (Fig 3B) and an even smaller amount in extracts of Ab+ CGD patients. Previously, it was concluded from Western blot analysis with a polyclonal rabbit antisera against the a subunit and from the absence of silver staining in the relevant region of SDS gels that the a subunit was absent in neutrophils of Xb CGD patients. Our results with MoAb 449 indicate that Xb CGD neutrophils do synthesize the a subunit of cytochrome b559. However, this a subunit is probably unstable owing to a defective synthesis of the b subunit. Surprisingly, a very small amount of the a subunit is also present in Ab+ CGD neutrophils, suggesting that even in these neutrophils some of this protein is produced. This finding might be related to our observation that a residual burst activity is present in these neutrophils, which is increased after in vitro treatment with interferon-γ.

The availability of MoAbs against both subunits of cytochrome b559 enabled us to monitor the presence of this complex during granulocytic maturation without the interference of other cytochromes. We showed that the steady-state levels of the proteins recognized by MoAbs 48 and 449 increase on granulocytic differentiation of HL-60 cells (Fig. 5). However, the 75-Kd glycoprotein present in cytochrome b559 of normal human neutrophils appears to be absent from HL-60 cells, even after DMSO treatment. This result suggests important differences in processing of this protein between differentiated HL-60 cells and normal human neutrophils.

An important conclusion can be drawn from the observation that MoAb 449 is not able to bind to neutrophils unless the cells are permeabilized (Fig 6). MoAb 449 does bind to purified plasma membrane vesicles in an ELISA or after Western blotting (D. Ambruso and D. Roos, unpublished observations), indicating that accessibility limits the binding of the antibody to cytochrome b559 present in the plasma membrane. Recently, MoAb 7D5, isolated by Nakamura et al., was shown to be directed against the a subunit of cytochrome b559 as well. MoAb 7D5 is able to bind to intact neutrophils. The discrepancy in binding to intact neutrophils between the two MoAbs is also observed when both are used in the same experiment (results not shown). This discrepancy shows that the epitopes recognized by MoAbs 449 and 7D5 are different and, moreover, that the a subunit of cytochrome b559 is a true transmembrane protein expressing epitopes for these antibodies on either side of the membrane.

Part of the cDNA encoding the low-mol-wt subunit of cytochrome b559 has been cloned with the help of MoAb 449. The sequence of the cDNA encoding the protein recognized by MoAb 449 (Fig 7) does not show clear homologies with other heme-containing proteins, but a stretch containing
His-94 does show some homology with the heme-bearing subunit I of cytochrome c oxidase. In addition, six of nine residues surrounding His-94 are also found in a stretch containing His-109 of cytochrome b55, an electron transport protein present in secretory vesicles of chromaffin cells. These similarities strongly suggest, but do not prove, a role of His-94 of the low-mol wt subunit in the noncovalent binding of the heme moiety in cytochrome b55. The sequence of the low-mol-wt subunit also contains a potential site for protein phosphorylation by protein kinase C. The stretch of several basic residues preceding the serine residue at position 63 of the protein is a common feature of substrates for protein kinase C. Indeed, Garcia et al have shown evidence for phosphorylation of the α subunit on neutrophil activation.

In conclusion, we succeeded in isolating two MoAbs against cytochrome b55 of human neutrophils. The first, MoAb 48, is able to bind to the high-mol-wt (or β) subunit of cytochrome b55 only on Western blots. The second, MoAb 449, binds to the low-mol-wt (or α) subunit under various conditions. Comparison with results obtained by Nakamura et al with another MoAb has established the transmembrane nature of this subunit.

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