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

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Monoclonal antibodies (MoAbs) were raised against cytochrome \textit{b}_{558}, a membrane-bound component of the NADPH:O2 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. The protein-defective in X-linked CGD has been cloned on the basis of its chromosomal location. The amino-acid sequence does not show similarities with other heme-containing proteins.

CYTOCHROME \textit{b}_{558} is a membrane-bound component of the NADPH:O2 oxidoreductase present in phagocytic cells.\textsuperscript{1} The NADPH:O2 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$_2^-$), a process generally known as the "respiratory burst" and essential for the killing of ingested bacteria. Inherited defects in the activity of the NADPH:O2 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 low absorbance spectrum, characteristic for cytochrome \textit{b}_{558}, cannot be detected in neutrophils of the patients. One form of CGD is linked to a gene defect on the X-chromosome,\textsuperscript{3} and the other is linked to an autosomal gene defect.\textsuperscript{4} The gene coding for the protein defective in X-linked CGD has been cloned on the basis of its chromosomal location.\textsuperscript{5} The amino-acid sequence of the putative gene product derived from the cDNA sequence does not show similarities with other heme-containing proteins.

Several groups of investigators have attempted to isolate cytochrome \textit{b}_{558} from human neutrophils, with different results.\textsuperscript{6,7} Recently, Segal\textsuperscript{8} and Parkos et al\textsuperscript{9} reported copurification of two proteins with different apparent molecular weights (molwts), both belonging to cytochrome \textit{b}_{558}, a 75- to 90-Kd glycoprotein and a 22- to 23-Kd protein. On the basis of amino-acid sequence identity\textsuperscript{10} and Western-blot analysis with polyclonal antisera,\textsuperscript{11} 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 \textit{b}_{558}-negative CGD might be due to a defect in the gene encoding the 22- to 23-Kd subunit.\textsuperscript{12}

To facilitate study of the structure of cytochrome \textit{b}_{558} and analysis of the defects observed in CGD, we decided to raise monoclonal antibodies (MoAbs) against the cytochrome \textit{b}_{558} complex of human neutrophils. Until now, only one MoAb (designated 7D5) has been described.\textsuperscript{13} We now report the isolation and characterization of two MoAbs specific for cytochrome \textit{b}_{558}. MoAb 449 binds to cytochrome \textit{b}_{558} under a variety of experimental conditions; it recognizes the low-mol-wt subunit of cytochrome \textit{b}_{558}. MoAb 48 binds only to detergent-solubilized cytochrome \textit{b}_{558} and recognizes the high-mol-wt subunit, as indicated by Western-blot analysis.

MATERIALS AND METHODS

Purification of cytochrome \textit{b}_{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 density 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$_4$Cl-KHCO$_3$-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 ×
10^4/mL were homogenized with a Sorvall Omnimixer (16,000 rpm) in a solution containing 0.34 mol/L sucrose, 1 mol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L EGTA, and 0.1 mol/L potassium phosphate (pH 7.4). A particulate preparation was fractionated by centrifugation of the homogenate (40,000 g for 30 minutes), and this fraction was subsequently stored at −70°C until further use. Cytochrome b$_{558}$ 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$^{1}$ by incubation for 20 minutes at 25°C. The Triton X-114 phase, containing all the cytochrome b$_{558}$, 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 b$_{558}$), and the suspension was stirred overnight. More than 90% of the cytochrome b$_{558}$ 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 b$_{558}$ (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 b$_{558}$ 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 (A448-A440). Cytochrome b$_{558}$ in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels was visualized by labeling the protein before electrophoresis by reductive methylation with 15$^{3}$C-formaldehyde in the presence of cyano-borohydride, according to the method described by Jentoff and Dearborn.$^{18}$ Protein content was measured with protein assay reagent containing bicinchoninic acid (Pierce Chemical, Rockford, IL) with albumin as a standard.

**Production of MonAbs.** BALB/c mice were injected intraperitoneally (IP) with 100 μL purified cytochrome b$_{558}$ (2 μmol/L, diluted 1:1 with Freund's complete adjuvant). A second and third injection with 40 μL purified cytochrome b$_{558}$ (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 b$_{558}$ 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.$^{19}$ Hybrids producing specific antibodies were selected with the enzyme-linked immunosorbent assay (ELISA) described below.

**ELISA for detection of antibodies against cytochrome b$_{558}$.** For selection of clones producing specific antibodies, an ELISA was developed with the antigen coated on the solid phase. Purified cytochrome b$_{558}$ (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 Na$_2$HPO$_4$, 1.3 mmol/L NaH$_2$PO$_4$, 100 mmol/L sucrose, 120 mmol/L HEPES, 1 mmol/L EGTA, 0.5 mmol/L PMSF, and 10 μmol/L leupeptin, pH 7.0). The postnuclear supernatant was centrifuged over a discontinuous sucrose gradient consisting of 2 mL 50% (wt/vol) sucrose and 1 mL 30% (wt/vol) sucrose for one hour at 100,000 g. The membranes at the 30% to 50% interface were harvested and frozen at −70°C until further use. These membranes represent a mixture of plasma membranes and specific granules.$^{21}$ After thawing, the membranes (adjusted to 0.5 mg protein/mL) were diluted 10-, 40-, 160-, and 640-fold in the ELISA coating buffer described above. All subsequent procedures were the same as those described above for the anticytochrome b$_{558}$ ELISA.

**Immunoblotting of cytochrome b$_{558}$.** Purified neutrophils were treated with DFP (described above) and subsequently lysed with a buffer (200 μL for 10$^6$ neutrophils) containing 1% (wt/vol) Triton X-100, 10 mmol/L HEPES, 3.5 mmol/L MgCl$_2$, 1 mmol/L PMSF, and 0.1 mmol/L leupeptin (pH 7.4). After centrifugation of the lysate (12,000g for 15 minutes), the supernatant was mixed with an equal volume of Laemmli sample buffer$^{22}$ containing 20% (wt/vol) SDS and incubated for 20 minutes at 60°C. A portion of the sample (equivalent to 2.5 x 10$^6$ neutrophils) was electrophoresed on a 5% to 15% or, in the experiments shown (see Fig 4), on a 12% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.45 μm, Schleicher & Schull, Dassel, FRG) by Western blotting (1A for 60 minutes) in a buffer (pH 8.2) containing 25 mmol/L Tris/HCl, 192 mmol/L glycine, 20% (vol/vol) methanol, and 0.1% (wt/vol) SDS. After blotting, the membrane was incubated for 30 minutes in Tris-buffered saline (10 mmol/L Tris-HCl, 150 mmol/L NaCl), pH 8.0) containing 1% (wt/vol) casein and 0.05% (wt/vol) Tween-20, and subsequently for 60 minutes with the appropriate antibodies (2 μg IgG/mL, diluted in Tris-buffered saline). After three washes with Tris-buffered saline containing 0.05% (wt/vol) Tween-20, alkaline phosphatase-conjugated goat anti-mouse Ig (Promega Biotec, Madison, WI) was harvested ten days after fusion, diluted fourfold in coating buffer containing 1% casein and incubated for one hour at 37°C with the antigen coated on the wells. The plates were then washed three times with PBS/Tween-20, and antibodies bound to the plates were detected by incubation for one hour at 37°C with rabbit anti-mouse-Ig, conjugated with horse-radish peroxidase (RAMPO) obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, diluted 1,500-fold in 0.2 mol/L Tris/HCl, pH 7.6). Thereafter, the plates were first washed with PBS/Tween-20, then with PBS, and finally with aqua dest. Color development was performed at room temperature by adding the substrates tetramethylbenzidine (Merck, Darmstadt, FRG; 5 mmol/L) and urea peroxide (Organon Teknika, Oss, The Netherlands; 2 mmol/L) in 100 μL sodium acetate (0.1 mol/L, pH 5.5). The reaction was terminated after ten minutes by adding 50 μL H$_2$SO$_4$ (2 mol/L), and absorbance at 405 nm of each well was determined in a Titertek spectrophotometer.
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 48 hours. The cells were centrifuged (800g for five minutes). The cells were washed twice with IMDM (pH 7.4), resuspended in incubation medium (107/mL) and fixed at room temperature by addition of an equal volume of 1% (wt/vol) paraformaldehyde. Five minutes after fixation, the cells were washed twice with PBS (containing 0.5% HSA and 0.1 mmol/L EGTA) and incubated for 30 minutes at room temperature with appropriate dilutions of antibodies. Subsequently, the cells were washed twice, FITC-labeled goat anti-mouse-Ig (CLB) was added, and incubations were continued for 30 minutes more. Fluorescence was measured with a FACSTAR fluorocytometer (Becton Dickinson, Palo Alto, CA).

To study the binding of antibodies to stimulated neutrophils, the cells (2.5 x 10^6/mL) were pretreated at 37°C with 1 mmol/L formyl-methionyl-leucyl-phenylalanine (fMLP) with cytochalasin B (5 µg/mL) added in the same incubation medium as described above, except that 1 mmol/L CaCl2 was added instead of EGTA. Control cells received vehicle only. Fixation of the cells was performed as described above for unstimulated cells.

**Table 1. Purification of Cytochrome b_{558} From 70 x 10^6 Neutrophils**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Total Content (nmol)</th>
<th>Specific Content (nmol/mg Protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate fraction</td>
<td>3,600</td>
<td>180</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>H2O phase</td>
<td>3,050</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Triton X-114 phase</td>
<td>550</td>
<td>180</td>
<td>0.32</td>
<td>100</td>
</tr>
<tr>
<td>After CM-Sepharose</td>
<td>44</td>
<td>120</td>
<td>2.72</td>
<td>67</td>
</tr>
<tr>
<td>Peak S-300</td>
<td>1.9</td>
<td>20</td>
<td>10.6</td>
<td>11</td>
</tr>
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</table>

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

**Isolation and sequencing of cDNA clone.** For immunoscreening, a λ-gt11 cDNA library constructed with mRNA from dimethylformamide (DMF)-treated human promyelocytic HL-60 cells was used. This library was a gift from Dr Stuart Orkin (Children’s Hospital, Harvard Medical School, Boston). Immunoscreening was performed with the Proteoblot 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 HincII 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...
MONOClonal antibodies against cytochrome b$_{558}$. MONOCLONAL ANTIBODIES AGAINST CYTOCHROME $b_{558}$

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 (described 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$^{8}$ 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 $b_{558}$

![Figure 2](image-url)

Fig 2. ELISA with neutrophil membranes and MoAb 449. Neutrophils of the following donors were coated on the solid phase: Xb- CGD (•••); control (••••); Xb- CGD (○○○); control (□□□□); Ab- CGD (•••); unaffected sister of Ab- CGD patients (••••); Ab- CGD (○○○); control (□□□□). Further experimental details are given in the Materials and Methods section.

![Figure 3](image-url)

Fig 3. Western blot of various neutrophil extracts incubated with MoAb 48 (A) or MoAb 449 (B). Neutrophil extracts from the following donors were loaded on an SDS-polyacrylamide gel: lane 1, control; lane 3, Xb- CGD; lane 4, Ab- CGD; lane 6, Ab- CGD. No samples were loaded in lanes 2 and 5. Blotting and incubation with antibodies was performed as described in the Materials and Methods section.

![Graphs](image-url)
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 \( \beta \) subunit\(^{16} \)) 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, 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 \( \sim 100 \) 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
threefold after stimulation of the cells with fMLP in the presence of cytochalasin B. 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 B13.9 directed against a 90-Kd protein present on the surface of neutrophils and on the interior of specific granules (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 X-gal1 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., 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.

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.

Fig 6. Flow cytometric determination of binding of MoAb 449 to intact neutrophils (A), permeabilized neutrophils (B), and activated neutrophils (C). Neutrophils were permeabilized with digitonin as described in the Materials and Methods section. (A and B) Binding of MoAb 449 (---) was compared with binding of an irrelevant antibody (— —) of the same subclass, IgG1. (C) Neutrophils were activated with fMLP with cytochalasin B added as described in the Materials and Methods section, and binding of MoAb 449 was compared with binding of a MoAb (B13.9) directed against a 90-Kd protein present in the plasma membrane and the specific granules of human neutrophils. Resting neutrophils incubated with MoAb 449 (----); activated neutrophils incubated with MoAb 449 (— —); resting neutrophils incubated with MoB 13.9 (-----); and activated neutrophils incubated with MoAb 13.9 (———).

**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. 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 \( \beta \)) 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 \( \alpha \)) 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 \( \beta \) subunit of cytochrome \( b_{558} \) in neutrophils from X-linked CGD patients, previously observed by other investigators. Some of these patients do, however, respond to treatment with interferon-\( \gamma \) with a partial recovery of respiratory burst activity, indicating that in these
cases the ability to synthesize (part of) the relevant protein is not completely absent. Neither is the subunit of cytochrome b558, probably reflecting the instability of this subunit when a complex with the γ subunit cannot be formed.

On a Western blot, MoAb 449 detects a small amount of α 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 antiserum against the α subunit14 and from the absence of silver staining of normal neutrophils that the α subunit is also present in neutrophils in Xb CGD patients. Our observation that even in these neutrophils some of this protein is present indicates that accessibility limits the binding of the antibody to cytochrome b559 present in the plasma membrane. Recently, MoAb 7D5, isolated by Nakumura et al.,15 was shown to be directed against the α 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 α subunit of cytochrome b559 is a true transmembrane protein expressing epitopes for these antibodies on either side of the membrane.

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. Ambrosio and D. Roos, unpublished observations), indicating that accessibility limits the binding of the antibody to cytochrome b559 present in the plasma membrane. 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.

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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

![Fig 7. Sequence of 680-bp insert encoding the 449 epitope. Isolation and sequencing were performed as described in the Materials and Methods section. The coding frame and the numbering of the deduced amino-acid sequence (shown above the nucleotide sequence) were derived from Parkos et al.16 Restriction site for HincII is underscored.](image-url)
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 b561, 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 b558. 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 b558 of human neutrophils. The first, MoAb 48, is able to bind to the high-mol-wt (or β) subunit of cytochrome b558 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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