Immunocytochemical Discovery of the 22- to 23-Kd Subunit of Cytochrome b_{558} at the Surface of Human Peripheral Phagocytes

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A monoclonal antibody raised against cytochrome b_{558} reacted specifically with the 22- to 23-Kd protein, the small subunit of this cytochrome. Cytochemical studies showed that the epitope was located on the surfaces of human phagocytes, such as neutrophils and monocytes. The small subunit of cytochrome b_{558} therefore, was expressed at least in part on the outer surface of these cells.

**MATERIALS AND METHODS**

**Materials.** Blood was drawn from normal subjects and patients with CGD after their informed consents were obtained. Genetic types of CGD were determined from the mode of heredity and from the complementation of the nitroblue tetrazolium (NBT) test in hybrid monocytes.

**Immunoblotting** of cytochrome b_{558}. An estimated 10 million neutrophils were purified by the method described previously and lysed with 1% (wt/vol) Triton X-100 in 0.2 mL of 10 mmol/L HEPES buffer (pH 7.4) containing 3.5 mmol/L MgCl$_2$, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mmol/L leupeptin. The lysate was incubated at 60°C for 20 minutes after addition of the same volume of 20% (wt/vol) sodium dodecyl sulfate (SDS) containing 5% mercaptoethanol. A portion of the sample was subjected to electrophoresis on an SDS–polyacrylamide gel (SDS-PAGE, 5% to 15%). Separated proteins were transferred to a nitrocellulose membrane (0.45 mm, Schleicher & Schuell, Dassel, W Germany) in 25 mmol/L Tris-HCI buffer (pH 8.2) containing 192 mmol/L glycine, 20% (vol/vol) methanol, and 0.1% (wt/vol) SDS at 1 A for 60 minutes. The membrane was soaked with 1% (wt/vol) casein and 0.05% (wt/vol) Tween 20 in 10 mmol/L Tris-HCI (pH 8.0) containing 150 mmol/L NaCl. This was then incubated with 7.5 μg of purified 7D5 (IgGI) in 5 mL of 10 mmol/L Tris-HCI (pH 8.0) and 150 mmol/L NaCl at room temperature. Alkaline phosphatase-conjugated goat antimouse IgG (Promega Biotec, Madison, WI) was then allowed to interact with the membrane for one hour, and the colors were developed by incubating with 0.45 mmol/L 5-bromo-4-chloro-3-indolyl phosphate and 0.38 mmol/L NBT for 30 minutes.

**Preparations for electron microscopy.** Peripheral leukocytes obtained from whole blood were fixed with 1% paraformaldehyde and 0.02% glutaraldehyde in Hanks’ balanced salt solution at 4°C for 60 minutes. Without any permeabilization of the membrane, the cells were incubated with the following reagents in this order: 1 mL of 2.5% nonimmune horse serum at 23°C for 60 minutes, antibody solution (20 μg IgGl/mL) at 4°C for 15 hours, biotinylated horse antimouse IgG antibody (Vector Laboratories, Burlingame, CA) at 23°C for two hours, a mixture of avidin and biotinylated peroxidase solutions (Vector) at 23°C for 70 minutes, and 50 mmol/L Tris buffer (pH 7.6) containing 6 mmol/L diaminobenzidine and 0.005% H$_2$O$_2$ at 23°C for 30 seconds. The cells were fixed with 1% paraformaldehyde and 0.02% glutaraldehyde in Hanks’ balanced salt solution at 4°C for 60 minutes. Without any permeabilization of the membrane, the cells were incubated with the following reagents in this order: 1 mL of 2.5% nonimmune horse serum at 23°C for 60 minutes, antibody solution (20 μg IgGl/mL) at 4°C for 15 hours, biotinylated horse antimouse IgG antibody (Vector Laboratories, Burlingame, CA) at 23°C for two hours, a mixture of avidin and biotinylated peroxidase solutions (Vector) at 23°C for 70 minutes, and 50 mmol/L Tris buffer (pH 7.6) containing 6 mmol/L diaminobenzidine and 0.005% H$_2$O$_2$ at 23°C for 30 seconds. The cells were fixed with 1% paraformaldehyde and 0.02% glutaraldehyde in Hanks’ balanced salt solution at 4°C for 60 minutes. Without any permeabilization of the membrane, the cells were incubated with the following reagents in this order: 1 mL of 2.5% nonimmune horse serum at 23°C for 60 minutes, antibody solution (20 μg IgGl/mL) at 4°C for 15 hours, biotinylated horse antimouse IgG antibody (Vector Laboratories, Burlingame, CA) at 23°C for two hours, a mixture of avidin and biotinylated peroxidase solutions (Vector) at 23°C for 70 minutes, and 50 mmol/L Tris buffer (pH 7.6) containing 6 mmol/L diaminobenzidine and 0.005% H$_2$O$_2$ at 23°C for 30 seconds. The cells were

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entangled with fibrin clots and embedded in Epon. Ultrathin sections were viewed on an electron microscope JEM-1200 EX (Jeol, Tokyo). Monoclonal antibody F4-11 (IgG1) against human carcinoembryonic antigen (Dr Kuroki, Fukuoka University) was used as a negative control.

RESULTS

SDS-PAGE of detergent-solubilized human neutrophils was performed and the proteins were transblotted to a membrane sheet. On the sheet, 7D5, a monoclonal antibody raised against human cytochrome b558 reacted with two adjacent proteins whose molecular masses were 22 and 23 Kd (Fig 1, lane 1). Both proteins were only just detected or were undetectable on the sheet when using the cytochrome-deficient neutrophils obtained from patients with X-linked CGD (Fig 1, lanes 2 and 3) or from an autosomal CGD (Fig 1, lane 4), respectively. These results indicate that 7D5 recognizes the small 22- to 23-Kd subunit duplex of the cytochrome. Although most of the large 70- to 92-Kd subunit of the cytochrome was transferred to the membrane sheet by blotting, it was not immunostained with 7D5.

Electron micrographs of the nonpermeabilized phagocytic cells that were immunostained with 7D5 are shown in Fig 2C and D. Electron-dense deposits of immunoperoxidase prod-
ucts were seen on the surface of neutrophils (Fig 2C) and monocytes (Fig 2D). In contrast, the cell surface of these cells was not immunostained with a control antibody, F4-11 (Fig 2A, B). More than 50 neutrophils and 20 monocytes were examined and all were found to be stained by 7D5. Dense deposits were also observed within the interior of the cells. However, the interiors were also stained with F4-11 (Fig 2A and C). Thus, endogenous peroxidase was responsible for these deposits.

**DISCUSSION**

In this communication we have demonstrated that the epitope of 22- and 23-Kd proteins to 7D5 is exposed on the cell surfaces of human neutrophils and monocytes. Both 22- and 23-Kd proteins are considered to be the small subunit of cytochrome b558 and they have an identical amino-terminal sequence (CG Teahan et al, personal communication, April 1988). These proteins are deficient in F4-11- and absent in autosomal, cytochrome b558-negative CGD neutrophils. The relative concentration of 22- to 23-Kd proteins varied from one experiment to another even if normal neutrophils of the same origin were used. Therefore, we cannot be certain that they directly correspond to the two spectroscopically distinguished species of the b-type cytochrome in neutrophils.

Although precautions were taken to prevent protein degradation in the experiments, a possibility remains that one of them was derived from the other by proteolysis during the preparation of samples.

Cytochrome b558 is known to have two subunits, 22 to 23 Kd and 70 to 92 Kd. It is uncertain which one of these two components of the cytochrome contains heme or whether both components contain heme. However, the small subunit of the cytochrome is more likely to carry heme since the amino acid composition of the large subunit that was predicted by nucleotide sequencing does not demonstrate any significant homology to that reported for purified b-type cytochrome or for other sequenced cytochromes. If the small subunit has heme, its surface location is consistent with the view that cytochrome b558 is the terminal oxidase in the O2-generating system. It is reported that almost all the oxygen consumed during the respiratory burst can be recovered as superoxide anions in the extracellular medium. The location of the hemoprotein on the surface is also consistent with the findings that the respiratory burst was reversibly inhibited by heme iron binding reagents such as pyridine and imidazole added to the medium. This inhibition was accompanied by a spectral change in the reduced cytochrome b558.

To our knowledge, cytochrome b558 is the only hemoprotein that is located on the cell surface of eukaryotic cells.

Finally, we must point out that the results found and reported here do not exclude the possibility that the same cytochrome or the small subunit exists also in granules. Present techniques do not allow us to investigate this since it is difficult to kill endogenous peroxidase completely without damaging the structure of the cells. However, it was established that the antigen was undetected within the interior of human alveolar macrophages permeabilized by freezing and thawing (unpublished observation, June 1987).

**REFERENCES**

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