Monoclonal Antibody 7D5 Raised to Cytochrome b$_{55}$ of Human Neutrophils: Immunocytochemical Detection of the Antigen in Peripheral Phagocytes of Normal Subjects, Patients With Chronic Granulomatous Disease, and Their Carrier Mothers

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We have established a monoclonal hybridoma clone that produces IgG1 against the cytochrome b$_{55}$ of human neutrophils. The antibody 7D5, secreted by the hybridoma, bound to solubilized cytochrome b of the neutrophils but not to other proteins such as hemoglobin, myeloperoxidase, and pig cytochrome P-450. Immunocytochemical studies of normal human peripheral blood showed that 7D5 bound to neutrophils and monocytes but not to lymphocytes or erythrocytes. The neutrophils of male patients but not of a female patient with chronic granulomatous disease lacked the antigen of 7D5 as well as the absorption spectrum for cytochrome b$_{55}$. A mosaic of the antigen-positive and -negative neutrophils was observed in mothers of the male patients. These biochemical and immunocytochemical results indicate that 7D5 is a specific antibody against cytochrome b$_{55}$ of human phagocytes.

MATERIALS AND METHODS

Materials. A mouse myeloma cell line, SP2/0, was kindly supplied by Dr K. Yagawa (Kyushu University, Japan). The monoclonal cell line that produces F4-11 (IgG1) against human carcinomaembryonic antigen (hCEA) was generously provided by Dr M. Kuroki of Fukuoka University, Japan.$^4$ Polyclonal IgG1 was purified from nonimmune mouse sera by Affi-Gel protein A of Bio-Rad (Richmond, CA).$^5$ Purified cytochrome P-450 of pig liver was a gift from Dr Y. Yasukochi (Tokyo Medical and Dental College). All plasticware except filtration plates were Falcon products (Becton Dickinson, Oxnard, CA). The filtration plates, Millititer-HA, were purchased from Millipore Corp (Bedford, MA). Polyethylene glycol 6000 and aminopterin are the products of Wako Chemicals (Osaka, Japan). The MonoAb-Screen System, which contained β-galactosidase-conjugated antimouse IgG, was purchased from Zymed Laboratories, Inc (San Francisco). Vectastain, an avidin-biotin-peroxidase complex kit, was obtained from Vector Laboratories, Inc (Burlingame, CA).

Blood. Blood was drawn into anticoagulant-containing tubes from normal subjects, patients with CGD, and their mothers after their and/or their parents' informed consents were obtained.

Preparation of the cytochrome b-rich fraction and solubilized cytochrome b. Human peripheral leukocytes were separated from acid-citrate-dextrose blood supplied by the Fukuoka Red Cross Blood Center according to the method previously described by Wakeyama et al.$^6$ The cytochrome b-rich fraction (C fraction) was prepared by the method of Gabig$^7$ without phagocytotic stimulation of the cells.

Phagocyte-specific cytochrome b was solubilized from the C fraction by 0.5% (wt/vol) Triton N-101 according to the method of Harper et al.$^12$ in 3-[N-morpholino]propanesulfonic acid (Mops) buffer (50 mmol/L Mops, pH 7.0, 100 mmol/L KCl, 5 mmol/L β-mercaptoethanol) at 4°C. The concentrations of cytochrome b in the C fraction and in the solubilized preparation were about 16 nmol/mL and 3 nmol/mL, respectively.

Immunization. An inbred BALB/c mouse was immunized three times, each time with 0.2 mL of a mixture containing 0.1 mL of C fraction and 0.1 mL Freund's complete adjuvant at intervals of 3 weeks. Ten days after the final immunization, spleen cells were

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separated and immunized in vitro by solubilized cytochrome b (6 to 60 pmol with 10 to 100 μg of Triton N-101 in 10 mL culture medium) according to the method of Luben and Mohler.13

Hybridization and screening. At the fourth day of the in vitro immunization, spleen cells (5 × 10⁸ cells) were harvested and hybridized by polyethylene glycol 6000 with logarithmically grown SP2/0 at a 6:1 ratio. The hybridized cells were washed once with RPMI 1640 (GIBCO, Grand Island, NY), suspended in medium containing 10% fetal calf serum, and seeded into 96-well microplates (Falcon 3072) at 2 × 10⁶ spleen cells per well as reported by de St Groth and Scheidegger.14 Eleven to 16 days after the seeding of cells, media of 1,645 wells with colonies resistant against hypoxantine-aminopterin-thymidine were screened on 96-well filtration plates, Millitititer HA, by an enzyme-linked immunosorbent assay with the MonoAb-Screen System. Each well of the plates had been coated with 25 μL of diluted C fraction that contained 2.9 pmol of cytochrome b. Each positive 499 colony was transferred to 24-well multwell plates (Falcon 3047) and grown in 1.2 mL of medium. The second screening was carried out by an absorption assay of solubilized cytochrome b.

Absorption assay of cytochrome b. Each hybridoma medium (usually 1 mL) was incubated for one hour with 10 μL of Sepharose 4B affinity gels. The ligand of the gels is rabbit IgG, which is monospecific to mouse IgG (heavy and light chains). After washing the gels four times with phosphate-buffered saline (PBS) containing 1 mmol/L NaCl and 0.1% Tween 20 and once with 0.1% Triton N-101–containing Mops buffer (25 mmol/L Mops, pH 7.0, 125 mmol/L KCl), the gels were incubated with 1.0 to 1.5 pmol of cytochrome b in 150 μL of the Mops buffer for 30 minutes at room temperature. After centrifugation of the gel suspension for four minutes at 10,000 g, 100 μL of the supernatant was transferred to a glass tube for the fluorometry of heme.

Fluorometric assay of heme. Heme in the supernatant was determined by the method of Morrison15 with a slight modification described by Sassa.16 To the supernatant, 0.4 mL of 2 mol/L oxalic acid was added, and the mixture was kept at 100°C for 30 minutes. After an addition of 0.7 mL of distilled water to the mixture, heme derivatives in the mixture were excited at 405 nm, and the consequent fluorescence emission was detected at 605 nm on a Hitachi 650-40 spectrofluorometer (Hitachi, Ltd, Tokyo).

Release of cytochrome b₅₅₈ from affinity gels. The cytochrome b bound to affinity gels was washed once to six times (Fig 1) or four times (Fig 2) with 1 mL Mops buffer (25 mmol/L Mops, 125 mmol/L KCl, pH 7.0) containing Triton N-101 (0.01% or 0.4%) and 1 mol/L NaCl. The trapped cytochrome was released from the gels by the addition of 150 μL of 0.1 mol/L glycine buffer (pH 2.5) with 0.1% Triton N-101 and 0.25% N-octylglucoside. The cytochrome recovered from the supernatant after centrifuging the gel suspension was assayed as described earlier.

Preparation of peripheral phagocytes for immunocytochemistry. A neutrophil-rich fraction was prepared from the mixture of 4 vol of peripheral blood with 1 vol of 3% (wt/vol) polyvinylpyrrolidone as reported by Wakeyama et al.18 A mononuclear cell fraction was prepared from whole blood on Conray-Ficoll as reported by Boyum.17 The fractions were washed four times with PBS and suspended in HEPES-saline buffer (17 mmol/L HEPES, pH 7.4, 5 mmol/L glucose, 1 mmol/L MgSO₄, and 0.5 mmol/L CaCl₂). The concentration of phagocytes was adjusted to 0.3 to 1.0 × 10⁶/mL.

Immunocytochemical demonstration of antigen of 7DS. Immuno-peroxidase staining by the avidin–biotin–peroxidase complex was carried out as described by Hsu et al19 with use of 0.02% 3,3′-diaminobenzidine. A drop of either cell fraction was put on a slide and incubated for 37°C for cell attachment. Unattached cells were rinsed from the glass with warm PBS. The remaining cells were air-dried, fixed in Hanks' balanced salt solution by 1% (wt/vol) paraformaldehyde and 0.02% (wt/vol) glutaraldehyde for 90 minutes at 4°C, and freeze-thawed once for penetration of the antigen through membranes. Nonspecific binding sites for immunoglobulin were blocked by the treatment of the cells with nonimmune horse serum for 20 minutes. About 100 μL of diluted culture medium containing monoclonal antibody 7DS (4 μg/mL) was dropped on the glass and kept overnight in a refrigerated-
tor. After thorough washing with PBS, the cells were incubated with biotinylated horse antirat IgG for one hour at room temperature. Endogenous peroxidase activity was denatured by the incubation of the cells in methanol with 0.01% H2O2 for 60 minutes at room temperature. They were thereafter incubated with the mixture of avidin and biotinylated peroxidase for 70 minutes and washed four times with PBS. Peroxidase activity was developed by treating the cells with 6 mmol/L diaminobenzidine and 0.005% H2O2 in 50 mmol/L Tris buffer (pH 7.6) for three to five minutes. The cells were counterstained with hematoxylin and embedded in paraffin. Photographs were taken using a Nikon FX-35A with a UFX-II exposure control unit on a Nikon FX-21 microscope.

Plain culture medium and F4-11, a monoclonal antibody against hCEA, were used for negative controls.

Statistical analysis. Means obtained from three or more values accompany the SD Student’s t test was used for confidence analysis.

RESULTS AND DISCUSSION

We prepared a C fraction of Gabig11 for in vivo immunization of a mouse and the solubilized preparation of the cytochrome for in vitro immunization of spleen cells and for binding assays of the cytochrome to monoclonal antibodies. The reduced minus-oxidized difference spectrum of the samples has the same absorption peaks at 429 nm, 530 nm, and 558 nm and a trough at 414 nm (data not shown), consistent with those of the cytochrome b found in rabbit granulocytes,2 porcine neutrophils,19 human peripheral phagocytes,3 and human colostral macrophages.40 Therefore, we concluded that the cytochrome was phagocyte specific. The solubilized preparation did not contain any myeloperoxidase because no significant absorption peak was observed at 475 nm in its spectrum. All the heme in the solubilized preparation is therefore derived from the cytochrome b. This conclusion is further supported by the observation that the concentration of heme in the solubilized preparation (3.3 nmol/mL) was essentially the same as that of the cytochrome (3.0 nmol/mL) assayed spectrophotometrically using 21 as a millimolar extinction coefficient. These observations justified the use of a fluorometrical assay of heme for the detection of the cytochrome in the preparation.

The second screening of the antibodies was done by mixing monoclonal medium and the affinity gels ligated with rabbit IgG against mouse IgG, centrifuging the gels, and incubating them with the solubilized cytochrome b. After the gels had been treated with the medium containing 7D5, the heme remaining in the supernatant was 0.09 ± 0.14 pmol, about 15% of the applied cytochrome. On the other hand it was 0.68 ± 0.13 pmol when the gels had been treated with the medium containing monoclonal antibody against a different protein of the C fraction of Gabig. The hybridoma secreting 7D5 was cloned twice by limiting dilution, and the resultant monoclonal hybridoma was ascended to secrete IgGl (data not shown). The antibody 7D5, secreted by the cloned hybridoma cells, was used in the present investigation.

The monoclonal antibody 7D5, but not nonimmune polyclonal IgG1, absorbed solubilized cytochrome b558 (Table 1). The 7D5 therefore specifically bound to the cytochrome of human neutrophils. To exclude the possibility that 7D5 binds to the cytochrome through other protein in micelles, we extensively washed the gels with Mops buffer (pH 2.4) gave a relatively broad band around 90 kilodaltons on sodium dodecylsulfate–polyacrylamide gel electrophoresis. Biochemical properties of the antigen will be presented in detail elsewhere.

The specificity of the antibody was shown further by the following experiments. (1) The antibody did not bind to phenobarbital-induced pig cytochrome P-450 (P > .05, Table 1). (2) The binding of 7D5 to the cytochrome was quantitative, as shown by the dose-response curve of the antibody (Fig 2). The cytochrome removed by 7D5 from the solubilized preparation was quantitatively recovered from the precipitate gels by acid treatments. When F4-11, a control monoclonal antibody against hCEA, was used, no cytochrome was removed from the supernatant or recovered from the precipitate gels. We therefore conclude that 7D5 is a monoclonal antibody against the phagocyte-specific cytochrome b of human neutrophils.

We used 7D5 for immunocytochemistry to demonstrate the presence of the antigen in human peripheral blood. The antigen was shown as brown deposits of polymerized diaminobenzidine in the cytoplasm of normal neutrophils (Fig 3A). Essentially the same deposits were also observed in monocyes (Fig 4A), in agreement with the presence of spectrophotometrically detectable cytochrome b.21 The deposits were granulated and diffusely distributed in the cells and overlapped with nuclei, thus suggesting that the antigen is located on lysosomes and cytoplasmic membranes. Further analyses including electron microscopy are, however, required to determine definitive subcellular localization of the antigen. No deposit was found in lymphocytes (Fig 3A, arrows) or in erythrocytes (Fig 3A, arrowheads). The latter observation indicates that hemoglobin, a b-type hemoprotein, is not antigenic to 7D5. A control monoclonal antibody (F4-11) did not give any deposits (Fig 3B) because of the absence of its antigen in these cells (M. Kuroki, personal communications). We could not completely denature the

Table 1. Amounts of Hemoprotein Remaining in Supernatant After Separation From IgGl-Treated Affinity Gels

<table>
<thead>
<tr>
<th>IgGl</th>
<th>Supernatant Heme (pmol)</th>
<th>Cytochrome b</th>
<th>Cytochrome P-450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgGl</td>
<td>0.85 ± 0.20</td>
<td>0.65 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>7D5</td>
<td>0.08 ± 0.06</td>
<td>0.47 ± 0.06</td>
<td></td>
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Each value is a mean ± SD obtained from three assays (see Materials and Methods).
MONOCLONAL ANTIBODY AGAINST CYTOCHROME b<sub>558</sub>

Fig 3. Immunocytochemical detection of the antigen of 7D5 in human peripheral neutrophils. (A) Neutrophils enriched from normal peripheral blood were attached to slides, fixed, freeze-thawed, incubated with culture medium containing 7D5, and finally counterstained by hematoxylin (for details see Materials and Methods). Arrows and arrowheads indicate lymphocytes and erythrocytes, respectively. (B) Same as A but immunostained with F4-11. (C) Same as A but immunostained with plain culture medium. A cell with brown deposits is an eosinophil that has been stained by residual endogenous peroxidase activity. (D) Neutrophils and a monocyte (inset) from a male patient with CGD were immunostained with 7D5 as in A. (E) Neutrophils from a female patient with CGD were immunostained with 7D5 as in A. Her cells were stained independently and look smaller than the others, possibly because of insufficient adhesion to glass. (F) Neutrophils from the mother of the patient shown in D were immunostained with 7D5 (Original magnification x 400).

endogenous peroxidase of eosinophils (Fig 3C) and failed to determine whether the cells had the antigen of 7D5.

The blood of patients with CGD and that of their mothers were also examined immunocytochemically. In contrast to normal phagocytes, neither neutrophils nor monocytes of a male patient were stained by 7D5 (Fig 3D). None of the cells was distinguishable from the normal phagocytes treated with culture medium (Fig 3C) or with F4-11 (Fig 3B). These two kinds of phagocytes of another five male patients (one reported in ref 22), were also not stained by 7D5. No characteristic spectrum of cytochrome b was observed in the neutrophils of those patients. Because myeloperoxidase was present in their cells, it should not have any antigenicity to 7D5. Neutrophils of a female patient with CGD were, however, clearly stained by 7D5 (Fig 3E). Her neutrophils exhibited a characteristic spectrum of the cytochrome (data not shown).

All mothers of the male patients thus far examined definitely had a mosaic of antigen-positive and -negative neutrophils in their blood. An example is shown in Fig 3F. This Lyon phenomenon indicates that the antigen is inherited on the X chromosome and is in good agreement with the findings that spectrophotometrically detectable cytochrome b is linked to that chromosome.<sup>5</sup> These biochemical and immunocytochemical results serve as two lines of confirmative evidence for 7D5 as a specific antibody against cytochrome b<sub>558</sub> of human phagocytes.

The present observations suggest that the absence of the cytochrome b spectrum from the neutrophils of the six male patients with CGD means the absence of holoprotein of the

Fig 4. Immunocytochemical demonstration of the antigen of 7D5 in normal peripheral monocytes. Mononuclear cells separated from peripheral blood were attached to slides. The cells were treated as in Fig 3 and immunostained with 7D5 (A) or F4-11 (B). (Original magnification x 500.)
cytochrome but not the presence of abnormal apoprotein with the mutation at the site for binding heme. The abnormal apoprotein can exist only when it is stable and has either two mutations, one at the heme-binding site and the other at the antigenic site for 7D5, or a mutation at the common site for binding heme and 7D5. The former is unlikely because the two mutations hardly occur by chance in all six genetically unrelated patients. The latter is also unlikely because 7D5 failed to bind not only to polymorphonuclear leukocytes of rabbits, guinea pigs, and rats (data not shown) but also to other human hemoproteins. These results indicate that the antigen of 7D5 is different from evolutionally conserved structures such as heme-binding site. The holoprotein of the cytochrome, therefore, must be absent in all neutrophils and monocytes of the six male patients with CGD and in all the antigen-negative cells of their mothers.

In summary, we obtained a monoclonal antibody, 7D5, that bound to phagocyte-specific cytochrome b. Our immunocytochemical studies with the antibody gave evidence for the lack of the cytochrome in peripheral neutrophils and monocytes of male but not of female patients with CGD and demonstrated the lyonization of the cytochrome in the cells of mothers of the male patients.

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