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Deficiency of cytochrome b_{558} in chronic granulomatous disease demonstrated by monoclonal antibody 7D5

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The production of superoxide anions (O_2^-) and the release of the anions into phagosomes are essential for the killing of bacteria by phagocytes in defense mechanism[1]. It is catalyzed by NADPH: O_2 oxidoreductase of cytoplasmic membranes of phagocytes. The activity of the enzyme is absent in phagocytes of patients with CGD [2]. Because the enzyme system is supposed to be composed of a few components, the disease should be caused by several etiology. Segal et al [3] suggested a novel cytochrome b which was first described by Hattori [4] as cytochrome b_{558} in 1961 to be involved in the activity. Its spectrum was absent or scarce in phagocytes of the patients with X-linked CGD [5], which is the major type of the disease.

A sensitive and quantitative detection of the cytochrome b is important for the definitive diagnosis of classical and variant types of CGD. The latter type is clinically not so severe and is seemed to be effectively treated with γ-interferon [6]. The detection should be done with a limited volume of blood because the patients are diagnosed early in their life.

We raised a monoclonal antibody against the cytochrome b of human phagocytes [7] which could specifically absorb solubilized O_2^--generating activity of human neutrophils [ref. 8 and unpublished observation by Murakami et al]. Here we used it for a sensitive and quantitative assay of the cytochrome b_{558} of phagocytes of CGD patients.

Materials and Methods

Monoclonal antibodies. More than 1,500 mononoclonal hybridomas were made from spleen cells of mice immunized with cytochrome b_{558}-rich human neutrophil membrane by fusing with SP 2/0. A hybridoma secreting 7D5 (IgG1) was cloned by several limited dilution procedures [7]. Culture medium of the clone was used. As a negative control IgG1, we used an anti-hCEA monoclonal antibody, F4-11, which was generously supplied by Dr. Kuroki (Fukuoka Univ., Fukuoka).

Immunostainings of peripheral phagocytes. Human peripheral phagocytes adhering to a slide glass were immunostained by 7D5 or F4-11 with the use of Vectastain (Vector Lab., Burlingame, CA) after denaturation of the endogenous peroxidase with 0.01% H_2O_2 in methanol [7].

Immunoelectron microscopy. Phagocytes fixed with 1% paraformaldehyde and 0.02% glutaraldehyde at 4 °C for 1 hr were immunostained as above and embedded in Epon. Ultrathin sections were observed on an electron microscope JEM-1200 EX (Jeol, Tokyo) [9].

Flow cytometry. Monocytes and neutrophils ($1-3.5\times10^6$ cells/ml) enriched on Percoll gradient were incubated with hybridoma culture medium containing either 7D5 or F4-11 at 6 °C overnight. These were washed once or twice with phosphatebuffered saline (PBS), incubated with FITC-labeled goat anti-mouse IgG $F(ab')_2$ (Cappel, Cooper Biomedical Inc., Malvern, PA) for 2 hrs at 6 °C and washed once with PBS. Flow cytometry was carried out on a Ortho Spectrum III (Ortho Diagnostic Systems, Westwood, MA) with a logarithmic or linear mode

Results and Discussion

Although normal human peripheral neutrophils were clearly immunostained with 7D5, those of patients with classical X-linked CGD were unstained with it [7]. Neutrophils of their mothers exhibited mosaicism, and were composed of two group, cells of one group were stained normally and those of the other were unstained (Fig. 1). Phagocytes of patients with autosomal recessive CGD were normally immunostained with 7D5. Immunocytochemical assay on a microscope is simple, sensitive and ivaluable as far as cytochrome b-positive and negative CGDs concern. But we discovered pa-

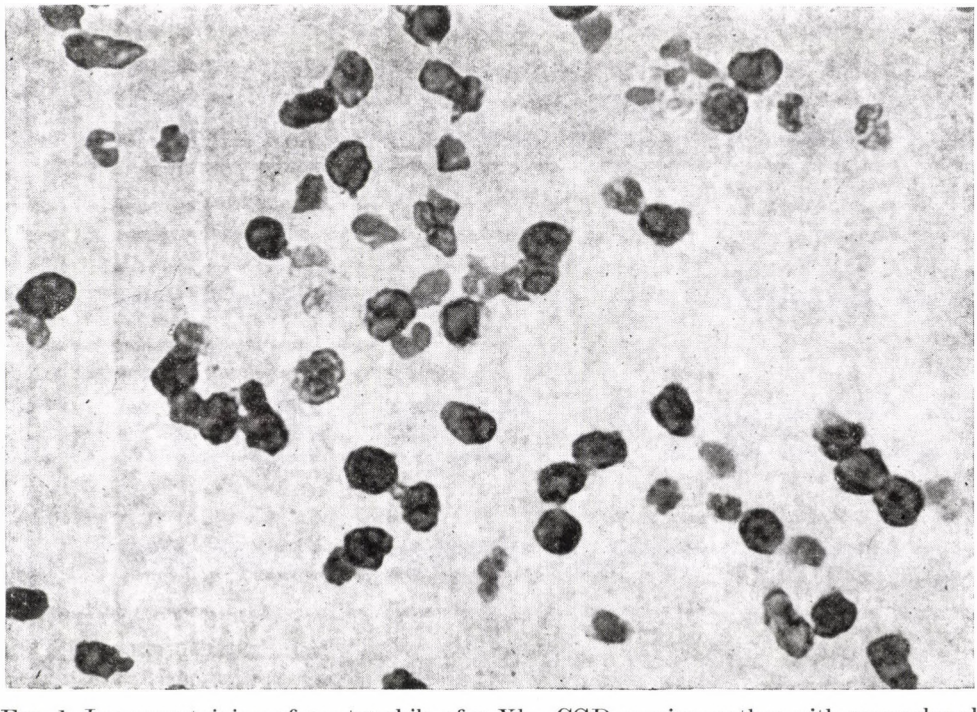

Fig. 1. Immunostaining of neutrophils of a Xb—CGD carrier mother with monoclonal antibody 7D5

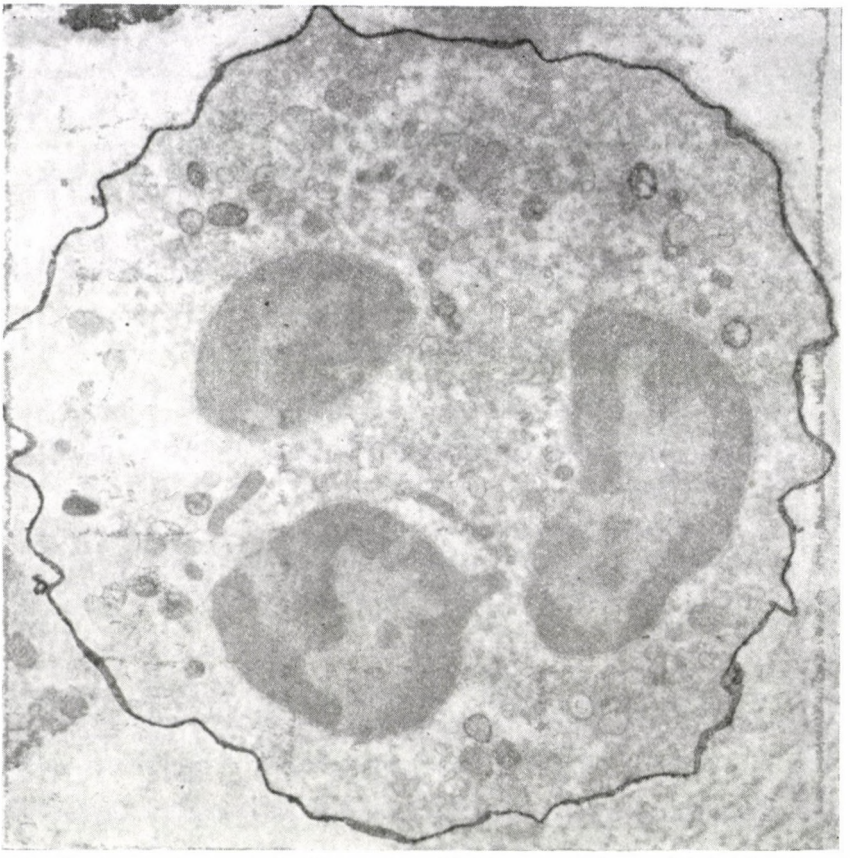

FIG. 2. An electron micropgraph of a neutrophil immunostained with 7D5. Deposits of oxidized diaminobenzidine were seen on the outer surface of cytoplasmic membrane. Deposits found inside the cell were produced by endogenous peroxidase activity

tients with neutrophils containing low but significant cytochrome b_{558} spectrum [10]. Although we occasionally succeeded in spectrophotometrically calculating the contents of the cytochrome in those cells, further accurate analysis was impossible because of limited numbers of available cells. In addition, the spectral assay of the cytochrome is greatly interrupted by contaminated hemoglobin even though it can be avoided to certain extent by the use of carbon monoxide [11].

We, therefore, established flow microfluorometry of phagocyte cytochrome b_{558} because the epitope of the cytochrome to 7D5 was found to locate on the surface of human phagocytes[9] (Fig. 2) and of B lymphocytes (K. Kobayashi et al & Maly et al, manuscripts in preparation). On the flow cytometry, 7D5-positive cells were separated from 7D5-unstained ones more clearly in monocyte fraction than in neutrophil fraction. So we used the monocyte fraction for the cytometry. Monocytes of some pa-

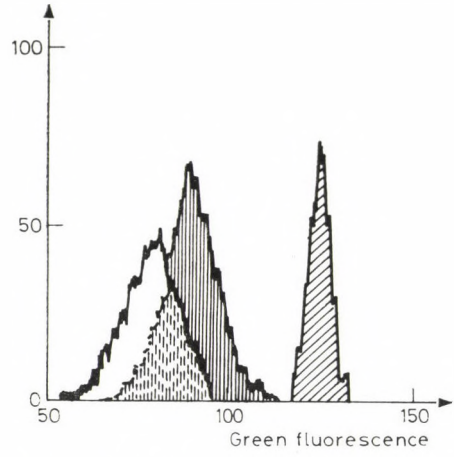

FIG. 3. Histograms of monocyte flow cytometry of a variant CGD patient. Open histogram, patient cells labeled with F4-11; closed histogram, patient cells labeled with 7D5; hatched histogram, cells of his father labeled with 7D5

tients definitely had low but significant amount of cytochrome b on the surface (Fig. 3). From our standard curve, the content of the cytochrome in the case shown in Fig. 3 was calculated to be about 14% of normal value which was comparable to that calculated from difference absorption spectrum (7–14%). This method required neither purified phagocytes nor abundant number of cells.

TABLE I

Classification of CGD

Chromosome	Cytochrome b_{558}		O_2^--generating activity	n
	spectrum	7D5 antigen		
X	—	—	—	6
	±	±	±	3
A	+	+	—	2
UC*	±	±	—	1

* Unclassified

We diagnosed 12 CGD patients and classified them into several groups by immunocytochemical and biochemical data (Table I). A half of patients belonged to classical X-linked cytochrome b-negative type, and one-fourth patients were X-linked variant CGD. We failed in classifying the last case of the table from family analysis of the cytochrome at protein level. His cells should be therefore analyzed genetically at a molecular level. We have experienced no definitive patient with autosomal cytochrome b--negative CGD in Japan.

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