Cytochrome *b* Deficiency in an Autosomal Form of Chronic Granulomatous Disease

A Third Form of Chronic Granulomatous Disease Recognized by Monocyte Hybridization

Ron S. Weening, Lucien Corbeel, Martin de Boer, René Lutter, Rob van Zwieten, Mic N. Hamers, and Dirk Roos

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology, and Department of Pediatrics, Academic Medical Center, University of Amsterdam, The Netherlands; Department of Pediatrics, Academic Hospital Gasthuisberg, University of Leuven, Belgium

Abstract

Three patients (two sisters and a brother) in one family are described with chronic granulomatous disease. The granulocytes of these patients did not respond with a metabolic burst to various stimuli and failed to kill catalase-positive microorganisms. The magnitude of the cytochrome *b* signal in the optical spectrum of the patients' granulocytes was <4% of the normal value, whereas the amount of noncovalently bound flavin in these cells was normal. The mode of inheritance of the genetic defect in this family is autosomal because the granulocytes of both parents (first cousins) and a nonaffected sister of the patients expressed 70–80% of the normal cytochrome *b* signal, showed low-normal or subnormal oxidative reactions during stimulation, and did not display mosaicism in the stimulated nitroblue-tetrazolium slide test. Somatic cell hybridization was performed between the monocytes from the affected boy in this family with monocytes from either a cytochrome *b*–negative male patient with X-linked chronic granulomatous disease or a cytochrome *b*–positive male patient with the classic autosomal form of this diseae. In both combinations, monocyte hybrids were observed with nitroblue tetrazolium reductase activity after stimulation with phorbol myristate acetate. This complementation of the oxidase activity required protein synthesis. Our results prove that the defect in this family is genetically distinct from that in the other two forms of chronic granulomatous disease. Moreover, our results also indicate that the expression of cytochrome *b* in human phagocytes is coded by at least two loci, one on the X chromosome and one on an autosome.

Introduction

Chronic granulomatous disease (CGD)[1] is a rare disorder in which the patients suffer from severe recurrent infections with bacteria and fungi, due to an inability of their phagocytes (neutrophils, eosinophils, and monocytes) to kill catalase-positive microorganisms (1–3). The defective killing of these microorganisms is caused by the failure of CGD leukocytes to produce sufficient amounts of superoxide and hydrogen peroxide during phagocytosis (1–3). The enzyme system responsible for this process is an oxidase located in the plasma membrane of the phagocytes. Although this oxidase system is usually inactive, it can be stimulated to generate O_2^- and H_2O_2 as a result of binding of opsonized particles or soluble stimuli to membrane receptors on the phagocyte surface (4–6).

Segal and coworkers (7) have reported that the optical spectrum of human neutrophils contains a signal of a *b*-type cytochrome. This signal is not detectable in the optical spectrum of the neutrophils from CGD patients with an X-linked inheritance of the disease and is present in subnormal amounts in the neutrophils of obligate heterozygotes of this type of CGD (8). When normal neutrophils are activated under anaerobic conditions by a soluble stimulus, e.g., phorbol myristate acetate (PMA), the *b*-cytochrome is reduced and instantaneously reoxidized on admittance of air to the cuvette (9). This reaction is not observed with neutrophils from patients with the (presumably) autosomal form of CGD (9), although these cells do contain the *b*-cytochrome signal. These observations indicate: (*a*) that the *b*-type cytochrome is part of the respiratory burst system of human neutrophils, and (*b*) that the X-linked form of CGD is correlated with the cytochrome *b* deficiency, whereas the autosomal form of CGD is correlated with a defect in the redox reactions with cytochrome *b*.

It is known, however, that the heterogeneity of CGD is more extensive than just the X-linked, cytochrome-*b*-negative and the autosomal, cytochrome *b*–positive forms. A family with an X-linked, cytochrome *b*–positive CGD patient has been reported (10). Moreover, "variant" forms of CGD, both X-linked and autosomal, have also been described in which the patients' phagocytes respond to some but not to all stimuli of the oxidase system (3). This heterogeneity of CGD may reflect the complexity of the oxidase system, in that the disease may become manifest not only when components of this enzyme system are missing or structurally altered but also when defects occur in the activation mechanism. The distinction between these different forms of CGD can be very difficult because heterozygotes have only been found in the X-linked forms of the disease, extreme lyonization may obscure the classification, and the families are often small and genetically noninformative.

Recently, we have described a method to solve this classification problem (11). When monocytes from an X-linked,

Address correspondence to Dr. Weening, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, P.O. Box 9190, 1006 AD Amsterdam, The Netherlands.

Received for publication 7 June 1984 and in revised form 15 November 1984.

J. Clin. Invest.

0021-9738/85/03/915/06 $1.00
Volume 75, March 1985, 915–920

1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; FAD, flavin adenine dinucleotide; FMLP, formyl-methionyl-leucyl-phenylalanine; NBT, nitroblue tetrazolium; PMA, phorbol myristate acetate; STZ, serum-treated zymosan.

cytochrome *b*–negative CGD patient are fused with monocytes from an autosomal, cytochrome *b*–positive CGD patient, the somatic cell hybrids express nitroblue tetrazolium (NBT) reductase activity in the presence of PMA, indicating that the oxidase activity is restored in the fused monocytes. This proves that these two forms of CGD represent genetically distinct abnormalities. We now present evidence for a third form of CGD detected with this fusion technique.

Methods

Patients. We have studied the phagocytes of six members of a Turkish family (Fig. 1), in which the parents are first cousins. The clinical details and some of the laboratory findings have been described previously (12). The eldest child (II1) has died at the age of 5 yr from generalized salmonella infection and has not been investigated by us. The next three children (II2, II3, and II4) show growth retardation, hepatosplenomegaly, periorificial dermatitis, moderately increased susceptibility to bacterial infections, and a tendency to inflammatory disease. II2 had a liver abcess and shows symptoms of Crohn's disease. II3 had two episodes of pneumonitis and shows signs of arthritis. II4 shows signs of colitis. The youngest girl (II5) has a normal height and weight and has not presented with any abnormal clinical findings. The mother (I2) has a low-normal height (P_3–P_{10}) and suffers from severe chronic polyarthritis. She has two sisters and four brothers who are in good health, as are their children. The father (I1) is in good health, as are his three sisters, one brother, and their children. Because these relatives reside in Turkey, we were unable to investigate their cells.

Histological studies in II2, II3, and II4 showed only in II3 some granuloma formation in several organs. In all three of these children pigmented histiocytes were found, probably as an aspecific sign of chronic infection, inflammation, or both. Parameters of humoral immunity showed high serum levels of IgG, IgA, IgE, and total complement in II2, II3, and II4. High amounts of IgE were also found in the serum of II5. Isohemagglutinin and viral antibody titers were normal in all subjects, as was the opsonizing capacity of the sera for *Staphylococcus aureus*. Lymhocyte functions were also normal (percentage of T and B cells, induration after intradermal injection of varidase or candida extract, proliferation in vitro after stimulation with phytohemagglutinin or allogeneic lymphocytes).

Chemotaxis of neutrophils in vivo and in vitro and phagocytosis of *S. aureus* and *Lactobaccilus acidophilus* in vitro in the presence of normal serum were normal. However, the neutrophils of the three affected children (II2, II3, and II4) had no killing capacity for *S. aureus* or *Candida albicans* in vitro, whereas the youngest child (II5) and both parents had neutrophils with normal activity. The bactericidal activity against *L. acidophilus* was normal in the two patients tested (II2 and II3). The oxidative metabolism of the patients' neutrophils was also clearly abnormal. During phagocytosis of serum-treated zymosan (STZ), oxygen consumption was not increased and hydrogen peroxide formation was not detectable when the neutrophils of the three affected children were tested, but were low-normal with the neutrophils of the other family members. Hydrogen peroxide production by monocytes (only tested in II3) was also undetectable. Reduction of NBT in the nylon column dye test was strongly decreased in the three affected siblings, but low-normal in the other family members. The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione peroxidase, glutathione reductase, and myeloperoxidase were normal in the neutrophil lysates of all family members.

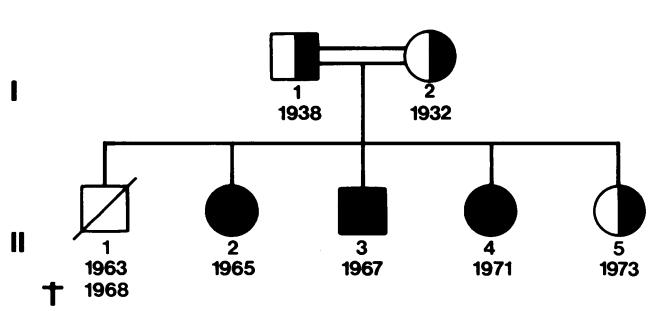

Figure 1. Pedigree of the family described in this article.

Materials. Percoll, Sepharose 4B, and Sulphopropyl Sephadex C-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Lucigenin (10,10′-dimethyl-9,9′-biacridinium dinitrate; Sigma Chemical Co., St. Louis, MO), PMA (Consolidated Midland Corp., Katonah, NY), calcium ionophore A23187 (Calbiochem-Behring Corp., San Diego, CA), formyl-methionyl-leucyl-phenylalanine (FMLP; Vega Biochemicals, Tucson, AZ), cytochalasin B (ICI Research Laboratories, Alderley Park, Cheshire, United Kingdom) and NBT (Sigma Chemical Co.) were dissolved in dimethyl sulfoxide and stored at −70°C. STZ was prepared as described before (13). Nuclear fast red (Merck & Co., Darmstadt, Federal Republic of Germany) was dissolved to 1 mg/ml in 5% (weight/volume) $Al_2(SO_4)_3$ solution of 70°C and filtered before use. Polyethylene glycol (mol wt, 4000) was obtained from Merck & Co.

Cell separation. All cell separations were performed under sterile conditions. Granulocytes were purified from citrated blood by centrifugation over Percoll with 0.5% (weight/volume) human albumin and 15 mM sodium citrate (specific gravity, 1.077 g/cm^3, 290 mosmol, pH 7.4), followed by NH_4Cl lysis of the erythrocytes in the pellet at 4°C (three times), essentially as described before (14). Monocytes were purified from the interface of the Percoll separation by elutriation centrifugation (15). The cells were suspended in incubation medium (138 mM NaCl, 2.9 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.5 mM glucose, and 0.5% [weight/volume] human albumin). The resulting cell suspensions were 90–95% pure.

Chemiluminescence assay. Mini vials (Packard Instrument Co., Downers Grove, IL) were filled with 1.95 ml of incubation medium, containing lucigenin (320 μM) and one of the following stimuli: STZ (1 mg/ml), PMA (160 nM), A23187 (10 μM), or FMLP (2 μM) with cytochalasin B (10 μg/ml). The vials were placed in polyethylene counting vials in a scintillation spectrometer with a controlled temperature of 37°C (Packard Tri-carb 3310, Packard Instrument Co.). The reaction was started by the addition of 5×10^4 granulocytes in 0.05 ml of incubation medium. Each vial was counted continuously for 6 min in the out-of-coincidence mode of the apparatus.

NBT slide test. NBT (50 mg/ml in dimethyl sulfoxide) was diluted ten times with phosphate-buffered saline (PBS; 140 mM NaCl, 9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4; pH 7.4) and pressed through a filter with pores of 0.45 μm. This solution was used only on the day of preparation. Granulocytes or monocytes (6×10^5 cells in 0.9 ml of incubation medium) were mixed with 0.1 ml of the NBT solution and incubated for 30 min at 37°C in the presence or absence of PMA (160 nM). Next, one sample was fixed in suspension for 5–10 min with paraformaldehyde (final concentration, 1%) and ~100,000 cells were spun on a glass slide with a Shandon Elliot Cytospin centrifuge (10 min, 600 rpm, room temperature). The slide was dried in air for about 5 min and stained for 10 min with nuclear fast red (0.1%, weight/volume, freshly filtered). Another cell sample (~4×10^4 cells) was first spun onto a glass slide, dried in air for at least 5 min, and stained with May-Grünwald solution (undiluted) for 5 min, followed by Giemsa solution (diluted 20 times in 10 mM phosphate buffer, pH 7) for 30 min. Both slide preparations were rinsed with tap water, air-dried, and embedded in malinol for microscopic examination. From each donor, 1,000 cells were scored as NBT-positive or NBT-negative in the nuclear fast red-stained preparations. Further differentiation in weakly, moderately, or strongly positive reactions and in cell type (neutrophil or eosinophil) was performed with the May-Grünwald/Giemsa preparations.

Determination of cytochrome b. Granulocytes (1.2×10^8) were solubilized for 10 min at 0°C in 1.5 ml of 2% (volume/volume) Triton X-100 in 100 mM potassium phosphate (pH 7.2). Nonsolubilized material was removed by centrifugation (20 min, 23,000 *g* at 4°C). To

eliminate contaminating hemoglobin, the cytochrome-*b*-containing supernate was incubated end-over-end for 15 min at 4°C with haptoglobin coupled to Sepharose 4B. 5 mg of haptoglobin (gift of Dr. M. Klein and Dr. L. Bernini, Sylvius Laboratories, University of Leiden, The Netherlands), coupled to 1 ml of swollen CNBr-activated Sepharose 4B, bound 5–8 × 10^{-8} mol of hemoglobin (i.e., in a 1:1 stoichiometry) but no cytochrome *b*. Subsequently, the Sepharose was removed by sedimentation. The reduced-minus-oxidized spectra of the extracts were recorded on a Cary-219 spectrophotometer (Varian Associates, Palo Alto, CA) with autobase-line correction, scanning speed of 1 nm/s and spectral bandwidth of 0.5 nm. Cytochrome *b* was reduced with a few grains of sodium dithionite. Myeloperoxidase, which interfered slightly with the spectrum of cytochrome *b*, was removed from the extracts of the patients' granulocytes by adsorbance for 20 min at 4°C on sulphopropyl Sephadex C-50, previously equilibrated with 100 mM potassium phosphate of pH 7.2 with 0.1% (volume/volume) Triton X-100 (16). For the quantitation of cytochrome *b*, absorbance coefficients of 106 $mM^{-1} \times cm^{-1}$ at 425 nm and 21.7 $mM^{-1} \times cm^{-1}$ at 558 nm (17) were used.

Determination of flavin. Noncovalently bound flavins were extracted from the membranes and cytoplasm of granulocytes. To this end, we first prepared cytoplasts (cell fragments composed of plasma membrane vesicles filled with cytoplasm but depleted of nuclei and granules) from human granulocytes, as described before (18). The cytoplasts (90 × 10^6) were centrifuged (800 *g*, 10 min, 4°C). The pellet was lysed by freezing and addition of 1 ml of distilled water to the frozen pellet. The lysed cytoplasts were centrifuged again (40 min, 160,000 *g*, 4°C). The membrane pellet and the supernate (cytoplasm) were extracted each with 1.0 ml of 5% (weight/volume) trichloroacetic acid. Denatured protein was spun down (10 min, 8,000 *g*, 4°C). To ensure complete removal of acid-extractable flavins, the pellets were treated with 1.0 ml of ice-cold 1% (weight/volume) trichloroacetic acid and centrifuged once more (10 min, 8,000 *g*, 4°C). The supernates from the membrane extractions were neutralized immediately and combined, as were the supernates from the cytoplasm extractions. The flavins were analyzed within 30 min (to limit hydrolysis of flavin adenine dinucleotide [FAD]) at pH 2.6 and 7.7, according to Faeder and Siegel (19). Flavin mononucleotide and FAD (Sigma Chemical Co.) were used as internal and external standards. More than 90% of the extracted flavin proved to be FAD (20). The extraction of noncovalently-bound flavins from whole granulocyte homogenates was essentially as described for the cytoplasts.

Monocyte hybridization. Freshly isolated monocytes (5 × 10^6 cells from each of two donors or 10^7 cells from one donor in 0.1 ml of Dulbecco's medium) were treated with polyethylene glycol (mol wt 4,000; final concentration 58%, weight/volume) in plastic round-bottom tubes (type 25200; Corning Glass Works, Corning, NY), for 1 min at 37°C. Thereafter, the cell suspension was diluted 20 times with Dulbecco's medium at room temperature and stored for 20 min on ice to recover. The cells were then centrifuged (250 *g*, 10 min, room temperature), resuspended in Iscove's modified medium with 10% (volume/volume) fetal calf serum to 10^6 cells/ml, and kept up to 40 h in 1-ml maintenance cultures in 2-ml wells of a tissue culture plate (no. 3524; Costar, Cambridge, MA). At the indicated times, the cells were collected, centrifuged (250 *g*, 10 min, room temperature), and resuspended in incubation medium for the NBT slide test. The total number of monocyte hybrids (large cells with two nuclei) was estimated from the May-Grünwald/Giemsa preparations, and the number of NBT-positive hybrids from the preparations stained with nuclear fast red. Per slide, all cells were screened (4–40 hybrids); per time point, 1–10 slides were examined.

Monocytes of patients II3 and II4 were fused with those of male CGD patients with the X-linked, cytochrome-*b*-negative form of the disease. Monocytes of patient II3 were also fused with those of a male, cytochrome-*b*-positive CGD patient with the classic autosomal recessive form of the disease. A patient was defined as having the X-linked form of CGD if: (*a*) the clinical history was suspect for CGD, (*b*) the granulocytes of the patient showed no respiratory burst upon stimulation with PMA or STZ, and (*c*) the granulocytes of the mother and/or sister had intermediate values in the metabolic assays and showed mosaicism in the NBT slide test. A patient was defined as having the classic autosomal recessive form of CGD if conditions (*a*) and (*b*) for X-linked CGD patients were fulfilled but no heterozygotes were detected in the family.

Results

Fig. 2 shows the results of the chemiluminescence assay. The granulocytes of the affected children (II2, II3, II4) failed to give a signal with STZ, PMA, or the calcium ionophore A23187. Only with FMLP plus cytochalasin B, a very low signal was found. The granulocytes of the unaffected child (II5) and of the parents gave intermediate signals between those of the affected children and the range of normal donors.

In the optical spectra of the granulocytes from the three affected children, the cytochrome *b* signal was hardly demon-

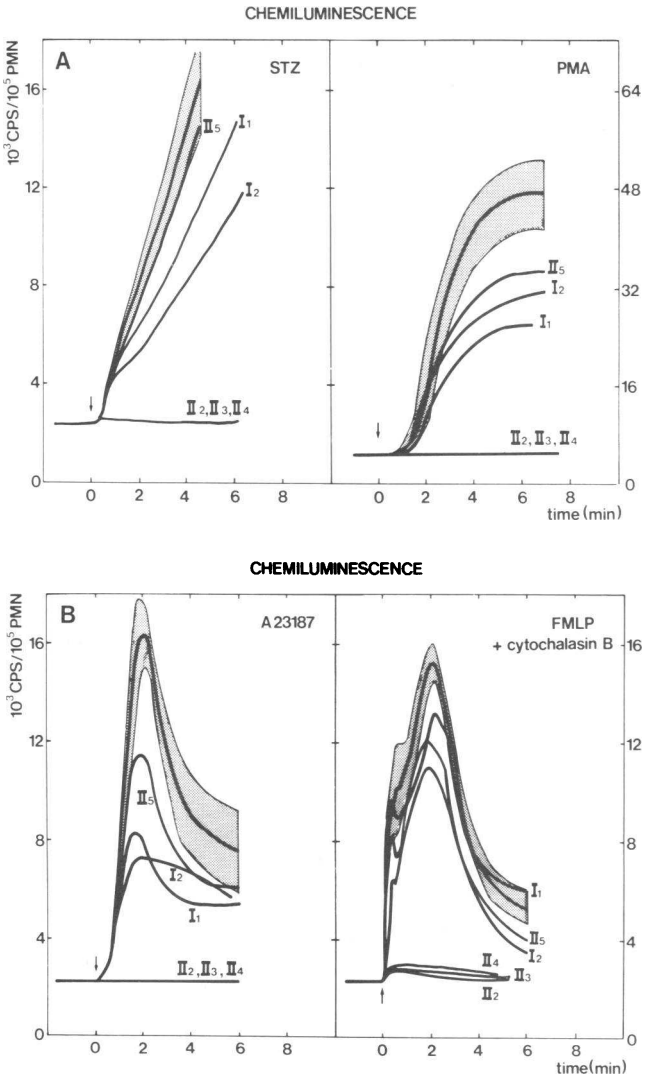

Figure 2. Chemiluminescence of granulocytes stimulated with (*A*) STZ or PMA, and (*B*) calcium ionophore A23187 or FMLP with cytochalasin B. For experimental conditions, see Methods. Arrows indicate the moment when a stimulus was added. The results obtained with granulocytes from six normal donors are given as a shaded area (mean±SEM). The reactions of the granulocytes from the family members are indicated by the pedigree symbols. Abscissa, reaction time in minutes; ordinate, chemiluminescence in 10^3 counts per second (CPS) per 10^5 neutrophils.

strable (Fig. 3). Table I shows that this represents 2–4% of the normal cytochrome *b* content. Subnormal amounts of cytochrome *b* were found in the granulocytes from the nonaffected child and the parents. The shape of the cytochrome *b* spectrum from the parents (not shown) and the unaffected child II5 (Fig. 3) was normal.

The amount of noncovalently bound flavins was determined in the membranes and cytoplasm of granulocyte cytoplasts. Table I shows that both values were normal in all family members. Furthermore, the amount of nonconvalently bound flavins was also determined in the homogenate of whole granulocytes from patient II2. This value, too, was normal; patient II2, 4.4 pmol/10^6 granulocytes; 8 controls, 4.6±0.55 pmol/10^6 granulocytes (mean±SD).

In the PMA-stimulated NBT slide test, a very low proportion of NBT-positive cells was detected in the preparations from patients II3 and II4, whereas no NBT-positive cells were observed in the preparation from patient II2 (Table I). All granulocytes of the other three family members showed a 100% positive NBT test. In contrast, obligate carriers of X-linked CGD showed a mosaic of strongly positive and completely negative granulocytes.

Table II shows the results of the monocyte fusion experiments. When the monocytes from two unrelated male patients

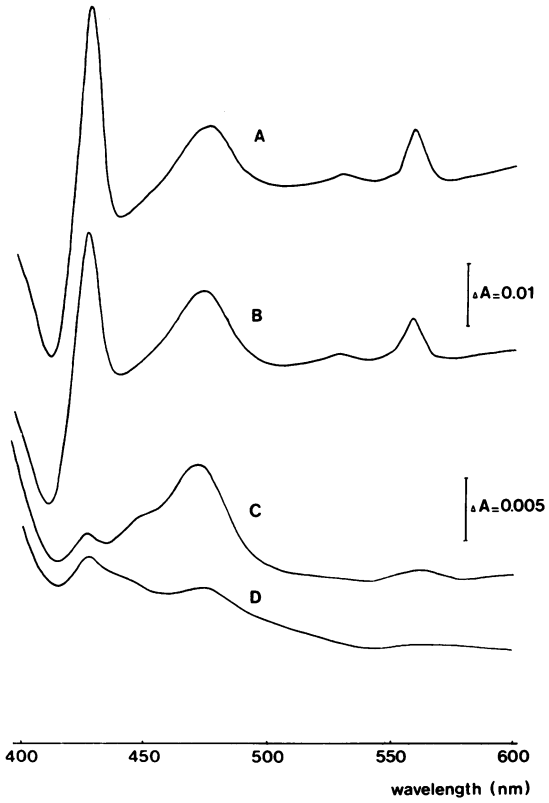

Figure 3. Reduced-minus-oxidized spectra of granulocyte extracts. For experimental conditions, see Methods. The spectrum of cytochrome *b* is characterized by the α-peak at 558 nm, the β-peak at 529 nm, and the γ-peak at 426 nm. The peak at 472 nm is caused by myeloperoxidase contamination. Extracts were made from the granulocytes of a healthy individual (trace A), the unaffected child II5 (trace B), and patient II2 (trace C). The granulocyte extract from patient II2 was also recorded after removal of myeloperoxidase with Sulphopropyl Sephadex C-50 (trace D). Traces A and B, absorbance scale marker 0.01; traces C and D, absorbance scale marker 0.005.

Table I. Cytochrome b, Nonconvalently Bound Flavins, and NBT Slide Test

Donor	Cytochrome *b**	Flavins‡		NBT slide test§
		Membranes	Cytoplasm	
Father I1	5.1	0.43	0.30	100
Mother I2	5.7	0.51	0.28	100
Child II2	0.3	0.39	0.37	0
Child II3	0.1	0.51	0.37	4‖
Child II4	0.2	0.39	0.27	3‖
Child II5	5.6	0.38	0.26	100
Controls	7.1±0.5	0.45±0.09	0.42±0.09	98–100
Obligate carriers of X-linked CGD	1.9–3.9	nt	nt	47–94

nt, not tested.
* Cytochrome *b* was measured in granulocyte extracts. Values in picomoles per 10^6 granulocytes. Control values were obtained with cells from five healthy individuals (mean±SD). For carriers of X-linked CGD (n = 5), the range is shown.
‡ Nonconvalently bound flavins were determined in extracts from membranes and cytoplasm of granulocyte cytoplasts. Values in picomoles per 10^6 cytoplasts. Control values as mean±SD of seven paired experiments with cells from seven healthy individuals.
§ NBT slide test of PMA-stimulated granulocytes. Values in percentage of cells showing formazan deposits. For the family members, the mean result of three tests is shown (with cells from three different blood donations). For controls (n = 10) and carriers of X-linked CGD (n = 5), the range is shown.
‖ Weakly positive.

(A and B) with the X-linked, cytochrome-*b*-negative form of CGD were fused, the resulting hybrids were all NBT-negative when tested with PMA. These hybrids remained unreactive to PMA when tested after 15 or 40 h of culture. Identical results were obtained with the monocytes from two unrelated, male patients (C and D) with the cytochrome-*b*-positive, autosomal recessive form of CGD. However, fusion of monocytes from patient A with patient C led to 32% PMA-reactive hybrids immediately after fusion. This percentage did not change significantly after 15 or 40 h of maintenance culture. Theoretically, the maximal percentage of NBT-positive hybrids is 50% because fusion between cells from the same donor occurs with the same probability as fusion between cells from different donors. Essentially, the same results were obtained when the monocytes of patient B were fused with those of patient D, and with the combination A × D (Table II). Nonfused monocytes of these four patients always remained PMA-unreactive in culture, in contrast with monocytes from healthy individuals which always remained NBT-positive. Fusion of monocytes from a single patient gave rise to homologous hybrids; with all four patients, these hybrids remained PMA-unreactive after culture. Homologous hybrids of normal monocytes remained PMA-reactive. These results indicate that patients A and B belong to one complementation group, whereas patients C and D belong to another complementation group.

We then purified the monocytes of patient II3 (a boy) and fused these cells with the monocytes of patient B or patient D (Table II). The combination II3 × B did not show PMA-reactive hybrids immediately after fusion. However, after 15 h

Table II. Expression of NBT-reductase Activity in Somatic Cell Hybrids of Monocytes from Five Male CGD Patients

Time after fusion	Percentage of NBT-positive homologous hybrids					Percentage of NBT-positive heterologous hybrids						
	A × A*	B × B	C × C*	D × D*	II3 × II3	A × B	A × C*	A × D*	B × D	C × D	B × II3	D × II3
h												
0–2.5	3 (n = 2)	0 (n = 3)	0 (n = 3)	2 (n = 3)	3 (n = 4)	0 (n = 2)	32 (n = 6)	30 (n = 4)	36 (n = 6)	0 (n = 2)	0 (n = 3)	15 (n = 6)
15	0 (n = 2)	0 (n = 3)	0 (n = 4)	0 (n = 2)	3 (n = 5)	0 (n = 1)	45 (n = 3)	44 (n = 10)	nt	0 (n = 1)	15 (n = 4)	54 (n = 2)
40	0 (n = 2)	0 (n = 3)	0 (n = 2)	0 (n = 2)	3 (n = 4)	0 (n = 1)	35 (n = 3)	22 (n = 7)	nt	0 (n = 1)	28 (n = 6)	46 (n = 6)

nt, not tested. NBT-positive hybrids are given as percentage of the total number of hybrids (n slides counted). The total number of hybrids was determined from slides stained with May-Grünwald/Giemsa. The results proved to be log-normally distributed and are therefore given as geometric means. Student's t test, corrected for separate variance estimates, was performed on the results obtained with the B × II3 and D × II3 fusion as compared with the values in the same row and in the same column. The results of this test ($0.0002 \leq P < 0.014$) are given in the text. A, B, C, D, and II3 indicate five different CGD patients; for details, see the text.
* Results taken from ref. 11.

of maintenance culture, 15% of the hybrids were PMA-reactive (difference with t = 0 h, P = 0.0002). After 40 h of culture, 28% of the hybrids were PMA-reactive (difference with t = 15 h, P = 0.009). The combination II3 × D showed 15% PMA-reactive hybrids immediately after fusion (difference with combinations A × C, A × D, and B × D at t = 0 h, P = 0.0004–0.014). This number increased to 54% after 15 h of culturing (difference with t = 0, P = 0.0008) and 46% after 40 h. The homologous hybrids of the monocytes from patient II3 persistently showed about 3% PMA-reactive cells.

In another experiment, the purified monocytes of patient II4 (a girl) were fused with those from a third male CGD patient with the X-linked, cytochrome-*b*-negative form of the disease. Again, PMA-reactive hybrids appeared in the course of 2 d of culture. However, when cycloheximide (1 μg/ml) was added directly after fusion, the hybrids remained PMA-unreactive, whereas addition of cycloheximide had no effect up to 40 h on the percentage of positive hybrids in the combination A × C (11). Hybrids of monocytes from patients II3 × II4 also remained PMA-unreactive.

Discussion

The patients described in this paper must be regarded as CGD patients because their phagocytes are functionally indistinguishable from the phagocytes of patients with CGD. However, the clinical histories of the patients are different from those of most other CGD patients, and more reminiscent of, although not identical with (12), those of the patients with lipochrome histiocytosis described by Ford et al. (21). Metabolically, the phagocytes of the patients here described are slightly different from those of classic CGD patients (very low activity in the NBT slide test with PMA and in the chemiluminescence assay with FMLP as well as a very low cytochrome *b* content). We have now found proof that the patients described in the present report belong to a separate type of CGD, with a different genetic mutation from either X-linked, cytochrome-*b*-negative or autosomal recessive, cytochrome-*b*-positive CGD.

There are strong indications that the defect in the presently described family is inherited in an autosomal way. Firstly, the amount of cytochrome *b* in the granulocytes was diminished in the nonaffected family members and hardly detectable in the patients. Secondly, low-normal or subnormal values were found with the granulocytes of the parents and their nonaffected daughter II5 in the oxygen consumption, hydrogen peroxide generation, NBT reduction (nylon column), and chemiluminescence assays. And finally, in the NBT slide test, no mosaicism was found in the granulocytes of the nonaffected girl or the parents.

Recently, a number of reports have described a diminished amount of flavins in CGD cells, both with and without cytochrome *b* deficiency (22–24). In the family here described, we found that the noncovalently bound flavin (mostly FAD) was present in normal amounts in the membranes and in the cytoplasm of granulocyte cytoplasts. Because cytoplasts generate similar amounts of superoxide and hydrogen peroxide per unit surface area upon stimulation as do intact granulocytes (18), those flavoproteins that are associated with the oxidase activity must be present in cytoplasts as well as in intact cells. Therefore, the fact that we found normal amounts of noncovalently bound flavins in the granulocyte cytoplasts from this family indicates that oxidase-associated flavoproteins were not deficient in this family. Moreover, the amount of nonconvalently bound flavins in the homogenate from whole granulocytes in patient II2 was also normal.

Because most CGD patients recorded at present either suffer from an X-linked, cytochrome-*b*-negative form of the disease or from a (presumably) autosomal recessive, cytochrome-*b*-positive form, the family here described (autosomal, cytochrome-*b*-negative) might belong to a third type of CGD. This notion was tested by monocyte hybridization. Indeed, as described before (11), we found complementation between the first two types of CGD but not within these groups, which proves that these two types are due to mutations at different genetic loci. We also found that the monocytes from the patients in the family described here gave rise to PMA-reactive hybrids with the monocytes from either of the two other types of CGD. This proves that the metabolic defect in this family is genetically different from that of both X-linked, cytochrome-*b*-negative and autosomal, cytochrome-*b*-positive CGD.

The granulocytes from patient II3, as well as the homologous monocyte hybrids, showed a low percentage of PMA-reactive cells, in contrast to the cells or hybrids from other CGD patients. This difference might be caused by the very small amount of cytochrome *b* still present in the cells of patient II3.

The lag-time observed in the appearance of PMA-reactive hybrids when the monocytes of II3 or II4 were fused with those of other CGD patients suggests that protein synthesis is required for the full expression of the NBT reductase activity in these hybrids. Indeed, cycloheximide prevented these hybrids from acquiring the ability to reduce NBT. In contrast, we found previously that protein synthesis is not required for the expression of the NBT reductase in hybrids of monocytes

from X-linked, cytochrome-*b*-negative CGD patients with monocytes from autosomal, cytochrome-*b*-positive CGD patients (11). In this latter combination, it appears that the components of the oxidase and of the activation mechanism for this system are donated directly by the fusing cells, and perhaps combine in the plasma membrane of the hybrids to form an active complex. The present results show that granulocytes or (cryopreserved) cytoplasts (25) should not be used for complementation studies because these cells lack protein synthesis.

Because cytochrome-*b*-negative phagocytes are found both in X-linked CGD and in the autosomal form of CGD described here, the expression of the *b*-heme protein is coded by at least two loci, one on the X-chromosome and one on an autosome. It may seem strange that the expression of one protein (cytochrome *b*) is coded by two different genetic loci. One must realize, however, that the expression of cytochrome *b* is measured spectrally. Absence of the *b*-heme optical signal may be caused by a number of different defects, e.g., a defect in the locus that codes for the protein of cytochrome *b*, a defect in the locus that codes for an enzyme needed for the heme incorporation into cytochrome *b*, a defect in the locus for an anchoring protein of cytochrome *b*, etc. It is conceivable, therefore, that protein synthesis is needed in the somatic cell hybrids between the two forms of cytochrome-*b*-negative CGD for the expression of NBT-reductase activity. We expect that this protein synthesis leads to expression of cytochrome *b* in these hybrids. Unfortunately, however, the fusion efficiency is so low that it is impossible to obtain enough hybrids to measure the *b*-heme signal.

It is clear that the complete unravelling of the molecular biology of the NADPH oxidase system of phagocytes requires much more work. It is also clear, however, that study of CGD patients, especially those with unusual abnormalities, yields important information about the composition of the oxidase system. The technique of monocyte hybridization can be of great value in these investigations.

Acknowledgments

This study was supported in part by grant no. 13-39-40 from the Foundation for Medical Research FUNGO, which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO), and by a grant from Beecham Farma B.V., Amstelveen, The Netherlands.

References

1. Klebanoff, S. J., and R. A. Clark. 1978. Chronic granulomatous disease. *In* The Neutrophil: Function and Clinical Disorders. Elsevier/North Holland Publishing Co., Amsterdam. 641–709.

2. Babior, B. M. 1978. Oxygen-dependent microbial killing by phagocytes. *N. Engl. J. Med.* 298:659–668; 721–725.

3. Tauber, A. I., N. Borregaard, E. Simons, and J. Wright. 1983 Chronic granulomatous disease: a syndrome of phagocyte oxidase deficiencies. *Medicine.* 62:286–309.

4. Wong, L., and J. D. Wilson. 1975. The identification of Fc and C3 receptors on human neutrophils. *J. Immunol. Methods.* 7:69–76.

5. Tauber, A. I., D. B. Brettler, E. A. Keunington, and P. M. Blumberg. 1982. Relation of human neutrophil phorbol ester receptor occupancy and NADPH-oxidase activity. *Blood.* 60:333–339.

6. Goetzl, E. J., D. W. Foster, and D. W. Goldman. 1981. Isolation and partial characterization of membrane protein constituents of human neutrophil receptors for chemotactic formylmethionyl peptides. *Biochemistry.* 20:5717–5722.

7. Segal, A. W., and O. T. G. Jones. 1978. Novel cytochrome *b* system in phagocytic vacuoles of human granulocytes. *Nature (Lond.).* 276:515–517.

8. Segal, A. W., A. R. Cross, R. C. Garcia, N. Borregaard, N. H. Valerius, J. F. Soothill, and O. T. G. Jones. 1983. Absence of cytochrome b_{-245} in chronic granulomatous disease: a multicenter European evaluation of its incidence and relevance. *N. Engl. J. Med.* 308:245–251.

9. Segal, A. W., and O. T. G. Jones. 1980. Absence of cytochrome *b* reduction in stimulated neutrophils from both female and male patients with chronic granulomatous disease. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 110:111–114.

10. Borregaard, N., A. R. Cross, T. Herlin, O. T. G. Jones, A. W. Segal, and N. H. Valerius. 1983. A variant form of X-linked chronic granulomatous disease with normal nitroblue tetrazolium slide test and cytochrome *b*. *Eur. J. Clin. Invest.* 13:243–247.

11. Hamers, M. N., M. de Boer, L. J. Meerhof, R. S. Weening, and D. Roos. 1984. Complementation in monocyte hybrids revealing genetic heterogeneity in chronic granulomatous disease. *Nature (Lond.).* 307:553–555.

12. Corbeel, L., R. S. Weening, D. Roos, S. Stadsbaeder, K. Geboes, L. Standaert, E. Eggermont, M. Casteels-Van Daele, B. Delmotte, and R. Eeckels. 1978. Familial deficiency of granulocyte bactericidal capacity associated with growth retardation. *Acta Paediatr. Belg.* 31:15–25.

13. Goldstein, I. M., D. Roos, H. B. Kaplan, and G. Weissmann. 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* 56:1155–1163.

14. Weening, R. S., D. Roos, and J. A. Loos. 1974. Oxygen consumption of phagocytizing cells in human leukocyte and granulocyte preparations: a comparative study. *J. Lab. Clin. Med.* 83:570–576.

15. Figdor, C. G., W. S. Bont, J. E. de Vries, and W. L. van Es. 1981. Isolation of large numbers of highly purified lymphocytes and monocytes with a modified centrifugal elutriation technique. *J. Immunol. Methods.* 40:275–288.

16. Wever, R., H. Plat, and M. N. Hamers. 1981. Human eosinophil peroxidase: a novel isolation procedure, spectral properties and chlorinating activity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 123:327–331.

17. Hamers, M. N., R. Wever, M. L. J. van Schaik, R. S. Weening, and D. Roos. 1980. Cytochrome *b* and the superoxide-generating system in human neutrophils. *In* Biological and Clinical Aspects of Superoxide and Superoxide Dismutase. *Dev. Biochem.* 11b:242–251.

18. Roos, D., A. A. Voetman, and L. J. Meerhof. 1983. Functional activity of enucleated human polymorphonuclear leukocytes. *J. Cell Biol.* 97:368–377.

19. Faeder, E. J., and L. M. Siegel. 1973. A rapid micromethod for determination of FMN and FAD in mixtures. *Anal. Biochem.* 53: 332–336.

20. Lutter, R., R. van Zwieten, R. S. Weening, M. N. Hamers, and D. Roos. 1984. Cytochrome *b*, flavins and ubiquinone-50 in enucleated human neutrophils (PMN cytoplasts). *J. Biol. Chem.* 259: 9603–9606.

21. Ford, D. K., G. E. Price, C. F. Culling, and P. Vassar. 1962. Familial lipochrome pigmentation of histiocytes with hyperglobulinaemia, pulmonary infiltration, splenomegaly, arthritis and susceptibility to infections. *Am. J. Med.* 33:478–489.

22. Cross, A. R., and O. T. G. Jones. 1982. The association of FAD with the cytochrome b_{-245} of human neutrophils. *Biochem. J.* 208:759–763.

23. Borregaard, N., and A. I. Tauber. 1984. Subcellular localization of the human neutrophil NADPH oxidase. *b*-Cytochrome and associated flavoprotein. *J. Biol. Chem.* 259:47–52.

24. Gabig, T. G., and B. A. Lefker. 1984. Deficient flavoprotein component of the NADPH-dependent O_2^--generating oxidase in the neutrophils from three male patients with chronic granulomatous disease. *J. Clin. Invest.* 73:701–705.

25. Voetman, A. A., A. A. M. Bot, and D. Roos. 1984. Cyropreservation of enucleated human neutrophils (PMN cytoplasts). *Blood.* 63:234–237.