The cytochrome *b*-558 molecules involved in the fibroblast and polymorphonuclear leucocyte superoxide-generating NADPH oxidase systems are structurally and genetically distinct

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We have demonstrated that human fibroblasts can release $O_2^{\cdot-}$ radicals by an NADPH oxidase system that appears to be functionally similar to the phagocytic system. Further analysis of these systems, however, with respect to the low-potential *b*-type cytochromes involved suggests that these two $O_2^{\cdot-}$ -generating systems are not structurally identical. Immunoblot analysis of fibroblast membranes with six different antibodies directed against both subunits of human neutrophil cytochrome *b*-558 indicated that the *b*-type cytochrome molecules involved in these systems were not identical. None of these anti-(neutrophil cytochrome *b*) antibodies recognized a similar cytochrome in fibroblast membranes, suggesting that the two cytochrome species are immunologically distinct. In addition, fibroblasts obtained from a patient suffering from X-linked chronic granulomatous disease (CGD) had a normal

cytochrome *b*-558 content compared with control fibroblast membranes, whereas the cytochrome *b*-558 concentration in polymorphonuclear leucocytes (PMNs) from this patient was decreased to 10% of that found in PMNs from healthy controls. Likewise, the stimulated $O_2^{\cdot-}$ release in PMNs from this patient was less than 10% of that in control PMNs, whereas the fibroblasts showed stimulated $O_2^{\cdot-}$ release rates that were indistinguishable from those of fibroblasts obtained from healthy persons. Since the genetic mutation responsible for this type of CGD results in the absence of cytochrome *b*-558 in PMNs, fibroblasts should be affected in the same way if both cytochrome species were identical. These results suggest therefore that the low-potential *b*-type cytochromes in PMNs and fibroblasts are structurally and genetically distinct.

INTRODUCTION

Many cell types have the ability to produce $O_2^{\cdot-}$, often in response to a specific stimulus (reviewed in [1]). Of these cell types, the phagocyte has been the focus of the majority of research on $O_2^{\cdot-}$ -generating systems (reviewed in [2–4]). Phagocytes (neutrophils, eosinophils and monocytes) generate $O_2^{\cdot-}$ in response to a variety of soluble and particulate stimuli and function as the body's first line of defence against infectious agents [2–4]. Recently it has also become established that non-phagocytic cells can also produce $O_2^{\cdot-}$, although the specific function(s) for the generation of this reactive oxygen species by these cells remains unknown. These cell types include B-lymphocytes, endothelial cells, fibroblasts, chondrocytes, epithelial cells, mesangial cells, smooth-muscle cells, cells of the corpora lutea, cells of the carotid body and platelets.

Activation of the phagocyte $O_2^{\cdot-}$ -generating system involves the translocation of cytosolic oxidase components [5,6] to the plasma membrane where they associate with a low-potential *b*-type cytochrome to form an electron-transport chain that transfers electrons from cytosolic NADPH to molecular oxygen [7–9]. The putative terminal component of this system is the low-potential *b*-type cytochrome known as cytochrome *b*-558 (also known as cytochrome *b*-559 or *b*₂₄₅) [8,10,11].

Human fibroblasts produce $O_2^{\cdot-}$ by an electron-transport system with similar functional characteristics to the NADPH oxidase system of phagocytes [12]. Recent studies by Meier et al. [12] demonstrated the presence of a flavoprotein of 45 kDa, which covalently binds diphenylene iodonium, and a low-

potential cytochrome *b*-558 in fibroblast membranes, although in lower concentrations than found in polymorphonuclear leucocytes (PMNs) or B-lymphocytes. However, the structural relationship of the component proteins of the phagocyte and fibroblast NADPH oxidase systems were not evaluated.

In the present paper, we analyse the cytochrome *b*-558 component of the neutrophil and fibroblast NADPH oxidase systems to determine their structural relationships. Using six different anti-(human neutrophil cytochrome) antibodies as structural probes, we show that the two cytochrome species are immunologically different. In addition, we analyse neutrophils and fibroblasts from healthy donors and from patients with X-linked chronic granulomatous disease (CGD) and show that the fibroblast cytochrome species is not structurally or functionally affected by the genetic defect of CGD that results in the loss of the neutrophil cytochrome *b*. These studies indicate that the two cytochrome species are structurally and genetically distinct.

MATERIALS AND METHODS

Materials

Established human fibroblast lines from a healthy donor and a patient suffering from Fanconi anaemia were obtained from the ATCC. Minimal essential medium (MEM) and trypsin/EDTA [0.125% (w/v)/0.01% (w/v)] were obtained from Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) was from Biochrom (Berlin, Germany) and Excell-300 medium from Isozol (Eching, Germany). Sterile plastic material for cell cultures was obtained from Nunc (Wiesbaden, Germany). Nitrocellulose membranes

Abbreviations used: PMN, polymorphonuclear leucocyte; CGD, chronic granulomatous disease; MEM, minimal essential medium; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; FMLP, *N*-formylmethionyl-leucylphenylalanine; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; FITC, fluorescein isothiocyanate; NBT, Nitroblue Tetrazolium.

for Western blotting were from Schleicher and Schüll (Dassel, Germany) and Immobilon PR nylon membranes were from Millipore (Göttingen, Germany). Cytochrome *c* grade III, peroxidase type III, 4,6-dioxyheptanoic acid, phorbol 12-myristate 13-acetate (PMA), *N*-formylmethionyl-leucylphenylalanine (FMLP), di-isopropyl fluorophosphate, SDS, 5-bromo-4-chloro-3-indolylphosphate (BCIP), phenazine methosulphate, phenazine ethosulphate, duroquinone, Mops, prestained molecular-mass markers, rabbit anti-mouse antibodies, goat anti-rabbit antibodies [fluorescein isothiocyanate (FITC)-conjugated] and rabbit anti-mouse antibodies (alkaline phosphatase-conjugated) were from Sigma (Deisenhofen, Germany). The Ca^{2+} ionophores, A23187 and ionomycin, and 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine were from Calbiochem (Frankfurt, Germany). 2-Hydroxy-1,4-naphthoquinone, anthraquinone 2,6-disulphonate, anthraquinone and 3-amino-1,2,4-triazole were from Aldrich (Steinheim, Germany), 3,6-diaminodurene was from Fluka, and Nitroblue Tetrazolium (NBT) and scopoletin were from Serva (Heidelberg, Germany). Interleukin-1 was obtained from Dainippon (Tokyo, Japan), tumour necrosis factor was kindly supplied by Schulze-Osthoff (Gent, Belgium) and superoxide dismutase was from Grünenthal (Aachen, Germany). Pyocyanine was synthesized by photo-oxidation from phenazine methosulphate [13], and diphenylene iodonium was synthesized from 2-iodobiphenyl by oxidation with peracetic acid [14]. The concentration of diphenylene iodonium solutions was determined photometrically with $A_{264} = 15.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. All other chemicals were from Merck (Darmstadt, Germany). The following antibodies directed against both subunits of the human neutrophil cytochrome *b*-558 were used: α -KQISNSES GPRG (residues 546–558), polyclonal large subunit antibody [15]; α -EARKKPSEEEAAA (residues 162–174), polyclonal small subunit peptide antibodies [16]; α -R3179, polyclonal small subunit antibody (holoprotein) [17]; 48, monoclonal large subunit antibody [18]; 449, monoclonal small subunit antibody [18]; and 7D5, monoclonal small subunit antibody to a surface epitope [19]. Plasma membranes of neutrophils were kindly supplied by Verhoeven (Amsterdam, The Netherlands).

Cell cultures

Primary cultures of human fibroblasts from two healthy people (one male aged 1 year and one female aged 36) and from a patient suffering from an X-linked CGD (male, 6 years of age) were established by the method of explant culture from tissues removed during surgery (informed consent was obtained before surgery) and propagated in MEM, supplemented with 10% (v/v) heat-inactivated FCS in 5% CO_2 in air at 37 °C. Two established fibroblast lines from a cell bank (ATCC) were treated like the cells derived from primary cultures. To avoid even low contamination of phagocytes, only cells of the 10th–20th passage were used for the experiments. Growth rate and $\text{O}_2^{\cdot -}$ production were similar to the fibroblast lines and remained constant between these passages. For determination of production of $\text{O}_2^{\cdot -}$ and H_2O_2 , the fibroblasts were cultured as monolayers on all four sides of glass cuvettes or on glass slides in four-multiplex chambers for staining with NBT and immunofluorescence determination of surface epitopes for binding antibody 7D5.

Determination of $\text{O}_2^{\cdot -}$ formation

Proliferating fibroblasts were cultured to confluency (5×10^4 cells/cm²) in cuvettes, as described, as monolayers on all four sides of glass cuvettes. The activity measurements were performed

in 2 ml of PBS supplemented with MgCl_2 (1 mmol/l), CaCl_2 (0.6 mmol/l) and glucose (10 mmol/l) (test buffer) at 37 °C. $\text{O}_2^{\cdot -}$ radical formation was determined photometrically (Uvikon 820; Kontron, Hannover, Germany) at 550 nm by measuring the reduction of cytochrome *c* (50 $\mu\text{mol/l}$) [20]. By addition of superoxide dismutase (100 nmol/l), it was confirmed that the reduction of cytochrome *c* was caused by $\text{O}_2^{\cdot -}$. Moreover the formation of $\text{O}_2^{\cdot -}$ was visualized by the reduction of NBT [21].

Inhibition of cytochrome synthesis

Cytochrome synthesis was inhibited by incubation of the fibroblasts in a cytochrome-free medium (Excell-300) supplemented with 4,6-dioxyheptanoic acid (150 $\mu\text{mol/l}$) [22] as inhibitor of cytochrome synthesis for up to 3 days.

Preparation of fibroblast membranes

Fibroblasts were detached with trypsin/EDTA (0.125%/0.01%, w/v), and 5 ml of MEM with 10% FCS was added per flask (80 cm²) to stop the reaction. The cells were sedimented by centrifugation (1000 *g*, 10 min), washed twice with PBS containing di-isopropyl fluorophosphate (2 mmol/l) as proteinase inhibitor at 0 °C, and sonicated on ice in four cycles of 15 s with 15 s interval with a Branson-185. Unbroken cells as well as nuclei and mitochondria were removed by centrifugation (3300 *g*, 10 min). The fibroblast membranes were precipitated by ultracentrifugation (105 000 *g* for 60 min) (crude membrane fraction). Pure plasma membranes were obtained as described by Jett et al. [23]. The detached fibroblasts were lysed with glycerine [30% (v/v) in PBS], and unbroken cells and nuclei were removed by centrifugation (10 min, 700 *g*, 4 °C) followed by a second centrifugation of the supernatant to remove cell fragments and mitochondria (10 min, 3300 *g*). The final supernatant was transferred to a centrifuge tube and 1 ml of a 38% (w/v) sucrose solution ($d = 1.1683$) was carefully added as a lower layer. After centrifugation (90 min, 95 000), the plasma membranes concentrated between the two phases (pure membrane fraction).

Determination of cytochrome *b*-558 content

Reduced-minus-oxidized difference spectra and spectra at known redox potential were recorded with a rapid-scanning split-beam spectrophotometer as described [24] or with UVikon-820 double-beam spectrophotometer containing a home-made stirring device. Reduction was performed anaerobically in 100 mmol/l KCl and 50 mmol/l Mops buffer at pH 7.0 with the following mediators: phenazine methosulphate (12.5 $\mu\text{mol/l}$), phenazine ethosulphate (12.5 $\mu\text{mol/l}$), pyocyanine (6 $\mu\text{mol/l}$), 2-hydroxy-1,4-naphthoquinone (12.5 $\mu\text{mol/l}$), anthraquinone-2,6-disulphonate (12.5 $\mu\text{mol/l}$), anthraquinone (12.5 $\mu\text{mol/l}$), 3,6-diaminodurene (12.5 $\mu\text{mol/l}$) and duroquinone (12.5 $\mu\text{mol/l}$). Reduction potential was decreased by the addition of small amounts of sodium dithionite solution and increased by addition of potassium ferricyanide solution, and determined anaerobically in a stirred cuvette fitted with platinum and calomel electrodes as described [25]. The absolute amount of the low-potential cytochrome *b*-558 at different redox potentials was calculated by measuring the absorption at 559 nm using $A_{559-540} = 21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

FACSscan and confocal microscopy

To test for surface epitopes of the small subunit of cytochrome *b*-558, fibroblasts were cultured on glass slides as described. The

Figure 1 Western-blot analysis of fibroblast membranes using anti-(cytochrome *b*-558) antibodies

Samples of partially purified neutrophil cytochrome *b*-558 (10 pmol) or neutrophil membranes (5 μ g), human fibroblast membranes of healthy donors (100 μ g), human fibroblasts of healthy donors cultured in a cytochrome-free medium (Excell-3000) containing 4,6-dioxiheptanoic acid for 2 days (100 μ g) or fibroblasts of a CGD patient were separated on 7–18% (**a–c**) or 6–12% (**d–g**) polyacrylamide/SDS gels and Western-blotted using six different anti-(human neutrophil cytochrome *b*-558) antibodies as indicated below for each blot (**a–g**). Samples of fibroblast membranes (100 μ g per lane) were also solubilized in each of the following: Triton X-100 (1%, w/v) as above, Triton X-100 (1%, w/v) plus SDS (10%, w/v), guanidine (4 mol/l) or urea (6 mol/l). These samples were then separated on a 10% acrylamide gel without SDS and blotted as described (**g**). Each of the blots is described below, including antibody used, specificity of the antibody and description of the sample in each lane of the blot. (**a**) α -KQS, anti-(human neutrophil large subunit) antibodies (polyclonal): lane 1, cytochrome *b*-558; lane 2, fibroblast membranes of healthy donors; lane 3, membranes of human fibroblast incubated with 4,6-dioxiheptanoic acid; lane 4, fibroblast membranes of a CGD patient. (**b**) α -EAR, anti-(human neutrophil small subunit peptide) antibodies (polyclonal): lane 1, cytochrome *b*-558; lane 2, fibroblast membranes of healthy donors; lane 3, membranes of human fibroblast incubated with 4,6-dioxiheptanoic acid; lane 4, fibroblast membranes of a CGD patient. (**c**) α -R3179, anti-[human neutrophil cytochrome *b* small subunit (holoprotein)] antibodies (polyclonal): lane 1, cytochrome *b*-558; lane 2, fibroblast membranes of healthy donors; lane 3, membranes of fibroblast incubated with 4,6-dioxiheptanoic acid; lane 4, fibroblast membranes of a CGD patient. (**d**) 48, anti-(human large subunit) antibody (monoclonal): lane 1, fibroblast membranes of healthy donors; lane 2, membranes of fibroblasts incubated with 4,6-dioxiheptanoic acid; lane 3, fibroblast membranes of a CGD patient; lane 4, neutrophil membranes. (**e**) 449, anti-(human small subunit) antibody (monoclonal): lane 1, fibroblast membranes of a CGD patient; lane 2, neutrophil membranes; lane 3, fibroblast membranes of healthy donors; lane 4, membranes of fibroblasts incubated with 4,6-dioxiheptanoic acid. (**f**) 7D5, anti-(human small subunit) antibody to a surface epitope (monoclonal): lane 1, cytochrome *b*-558; lane 2,

subconfluent cells were washed with warm PBS, incubated twice for 10 min with BSA solution (1% in PBS) to decrease non-specific binding, washed with warm PBS and incubated for 4 h with antibody 7D5. Antibodies not bound to the cells were removed by washing with PBS before an FITC-conjugated anti-mouse or anti-rabbit antibody was added. The binding of the antibody was determined in a FACScan (Becton Dickinson, Heidelberg, Germany) or observed in a confocal microscope (Zeiss, Göttingen, Germany).

Western blotting

Fibroblast membranes, prepared as described above, were solubilized with 1% (w/v) Triton X-100 in Hepes buffer (10 mmol/l, pH 7.4), and centrifuged (45 min, 100 000 *g*). The Triton X-100-soluble supernatant and insoluble pellet (resuspended in Triton solubilization buffer to a similar volume as the supernatant) were mixed 1:1 with Laemmli sample buffer, containing 10% (w/v) SDS and incubated for 60 min at room temperature. The samples were then separated by SDS/PAGE (7–18% (w/v) gradient gels for blotting with α -KQISN-SESGPRG, α -EARKKPSEEEAAA and α -R3179 antibodies or 6–12% (w/v) gradient gels for blotting 48, 449 and 7D5 antibodies). For further testing of 7D5, samples were treated alternatively with 4 mol/l guanidine or 6 mol/l urea and separated on 10% (w/v) acrylamide gels (without SDS). The separated proteins were then blotted on to nitrocellulose or immobilon PR membranes using standard methods. Non-specific binding of the antibodies was decreased by preincubation of the transfers in PBS containing BSA (1%, w/v) or non-fat milk powder (1%, w/v) for 1–3 h. After being washed, the transfers were incubated with the anti-cytochrome antibodies for 3 h at room temperature or overnight at 4 °C. Unbound antibodies were removed by washing with PBS containing 0.2% Tween 20, and an alkaline phosphatase-conjugated secondary antibody was incubated with the transfers for 1 h at room temperature. The blots were then developed using BCIP and NBT as substrate. A rabbit anti-mouse secondary antibody was used for the monoclonal antibodies tested and a goat anti-rabbit secondary antibody was used for the polyclonal antibodies tested.

RESULTS

Studies with antibodies directed to surface epitopes of the small subunit of neutrophil cytochrome *b*-558

The antibody 7D5 [19] was tested for binding to surface epitopes of cytochrome *b*-558 on native adherent fibroblasts (four lines) using confocal microscopy as described. It failed to bind to a surface epitope. To confirm this result, fibroblasts were also detached and analysed by FACScan. FACScan analysis of antibody-treated cells showed no shift compared with control fibroblasts not treated with the antibodies (not shown).

To examine this result and compare structural characteristics of fibroblast and neutrophil cytochrome *b*-558, fibroblast membranes were analysed by Western blotting with a number of previously characterized anti-(human neutrophil cytochrome *b*)

fibroblast membranes of healthy donors; lane 3, membranes of human fibroblast incubated with 4,6-dioxiheptanoic acid; lane 4, fibroblast membranes of a CGD patient. (**g**) 7D5, anti-(human small subunit) antibody to a surface epitope (monoclonal): fibroblast membranes solubilized with Triton X-100 (lane 1), Triton X-100 and SDS (lane 2), guanidine (lane 3) or urea (lane 4). Std = standard in all blots. The blots were representative of at least two experiments. In addition, further experiments analysing fibroblast membranes at up to 1 mg per lane gave identical results in the blots (**a–g**) (not shown). No differences were observable between the fibroblasts used including those derived from a CGD patient (X-linked form).

Figure 2 Quantitative determination of the stimulated $O_2^{\cdot -}$ release from fibroblast lines derived from a CGD patient (X-linked) and healthy donors

Fibroblast lines derived from a CGD patient ($\circ$) and from healthy donors (Δ) were cultured in cuvettes and the stimulated $O_2^{\cdot -}$ release was determined as described in the Materials and methods section. The following stimulants were used: 1, Ca ionophores (A23187 or ionomycin, each $10 \mu\text{mol/l}$); 2, 1-O-alkyl-2-acetyl-sn-glycyl-3-phosphorylcholine ($10 \mu\text{mol/l}$); 3, interleukin-1 (100 nmol/l); 4, tumour necrosis factor (100 nmol/l); 5, FMLP ($0.5 \mu\text{mol/l}$); 6, PMA ($2 \mu\text{mol/l}$). Data represent means $\pm$ S.D. of at least four different experiments.

antibodies. Polyclonal antibody α -KQISINSESGPRG, directed against residues 546–558 of the large subunit of the neutrophil cytochrome *b*-558 [15], as well as antibodies α -EARKKP-SEEEAAA, directed against residues 162–174 of the small subunit [16], and α -R3179, directed against the whole small subunit protein [17], showed no reaction with fibroblast membranes up to concentrations of 1 mg of protein per lane (Figures 1a–1c). The same results were obtained with the Triton X-100-soluble or Triton-insoluble fraction of fibroblast membranes.

Monoclonal antibodies 449, directed against the human neutrophil cytochrome *b* small subunit [18], and 48, directed against the large subunit [18], showed several non-specific bands with the fibroblast membranes over a wide molecular-mass range in Western blots (not shown), comparable with the Western blots obtained by Radeke et al. [26] for human mesangial cells. Bands were also observable in the molecular-mass range of both subunits of the neutrophil cytochrome *b*-558. However, Western-blot analysis of highly purified fibroblast membrane fractions with antibody 449 or 48 showed no staining, even at concentrations up to 1 mg of protein per lane, indicating that the bands observed with partially pure membranes were due to non-specific staining of proteins other than cytochrome *b*-558. The same result was observed when milk powder was used instead of BSA to block non-specific binding, i.e. no antibody staining was observed with the fibroblast membranes up to concentrations as high as 1 mg of protein per lane (Figures 1d and 1e), whereas the neutrophil membranes gave a strong positive reaction with both antibodies.

Western blotting with monoclonal antibody 7D5 [19] also gave no detectable staining with fibroblast membranes (Figure 1f). The procedure used for the preparation of the fibroblast membranes followed exactly the method used by Nakamura et al. [19] for PMNs, which indeed showed a positive reaction with the antibody. As Radeke et al. [26] suggested that SDS might prevent a reaction of this antibody with the cytochrome *b*-558 of PMNs and fibroblasts in dot-blots, we also solubilized the fibroblast membranes using Triton X-100, guanidine and urea (each 1–10%, w/v, in PBS) without further treatment with SDS (20%, w/v) as described by Nakamura et al. [19]. The

supernatants of each fraction were separated by SDS/PAGE or on a native gel and Western-blotted. Again, no reaction of the fibroblast membranes with antibody 7D5 was observable after Western blotting and staining with BCIP/NBT in the fractions treated with SDS or Triton X-100. However, after treatment with guanidine (4 mol/l for 1 h at room temperature) or urea (6 mol/l for 1 h at room temperature) a weak band was observable in the range of 80–85 kDa after the membranes had been developed for 4 h with BCIP/NBT (Figure 1g), whereas the specific reactions of the antibodies with the cytochrome *b*-558 in PMNs were observable after 30–60 min.

Determination of the stimulated $O_2^{\cdot -}$ production and concentration of cytochrome *b*-558 in fibroblasts of CGD patient (X-linked form)

The Western-blotting results described above suggested that fibroblasts and neutrophils possess immunologically distinct forms of cytochrome *b*-558. To confirm this conclusion and to determine whether these cytochromes were also genetically unrelated, PMNs and fibroblasts from a patient suffering from the X-linked form of CGD were studied. {The patient, an 8-year-old boy, was admitted to the Medical School, Hannover with liver abscesses. Analysing the PMNs by cytochrome *c* reduction assay for $O_2^{\cdot -}$ formation, flow cytometry using dihydro-rhodamine 123 for H_2O_2 production [27] and staining of membrane components of the NADPH oxidase with the monoclonal antibody 7D5, the diagnosis was established. Additionally, on analysing the PMNs of the mother, two populations (one normal and one showing the same features as the patient's PMNs) could be determined by the flow-cytometric methods indicating the X-linked trait of inheritance of the CGD.} The PMNs of this patient showed very low stimulated rates of $O_2^{\cdot -}$ production after treatment with PMA ($2.5 \mu\text{mol/l}$): $0.15 \text{ nmol } O_2^{\cdot -}/\text{h per } 10^6 \text{ cells}$ compared with control PMNs from normal healthy donors releasing $5\text{--}7 \text{ nmol } O_2^{\cdot -}/\text{h per } 10^6 \text{ cells}$. Moreover, FACScan analysis of these CGD PMNs after staining with antibody 7D5 gave only a slight but detectable shift compared with PMNs obtained from healthy donors. In contrast, the stimulated release of $O_2^{\cdot -}$ and H_2O_2 from the CGD fibroblasts was not different from those measured in fibroblasts obtained from healthy donors (Figure 2). CGD fibroblasts showed exactly the same rates of $O_2^{\cdot -}$ production as control fibroblasts after stimulation with Ca^{2+} ionophores (A23187 or ionomycin), PAF, cytokines (interleukin-1 or tumour necrosis factor), PMA or FMLP. Fibroblasts obtained from this CGD patient or from healthy donors also showed a similar distribution of Blue Formazan on the surface of the cells after stimulation with the substances mentioned above (Figure 3). The $O_2^{\cdot -}$ release was inhibited by diphenylene iodonium, which is an inhibitor of NADPH oxidase in PMNs [28], but not by rotenone, azide, cyanide or allopurinol. On incubation of the fibroblast cultures in the presence of 4,6-dioxyheptanoic acid ($150 \mu\text{mol/l}$) as inhibitor of cytochrome synthesis, in a cytochrome-free medium, the stimulated $O_2^{\cdot -}$ release decreased within 2 days to less than 10% and was no longer detectable after 3 days. Here again no difference was observable between the fibroblast lines derived from the CGD patient and healthy donors.

Analysis of the low-potential cytochrome *b*-558 content of these cells showed that the absolute amount of the cytochrome *b*-558 in fibroblast membranes was 10 pmol/mg of protein as determined photometrically by redox titration, which is exactly the same concentration as is present in fibroblasts obtained from healthy donors [12]. In contrast, the PMNs obtained from this patient possessed a decreased amount of cytochrome *b*-558 compared with healthy donors (approximately 10%).

Figure 3 Staining of fibroblasts from a CGD patient (X-linked) and healthy donor for O_2^- release with NBT

Fibroblasts of a CGD patient (a) and a healthy donor (b) were cultured and stained for O_2^- release with NBT as described in the Materials and methods section after stimulation with tumour necrosis factor (100 nmol/l). The cells were incubated at 37 °C for 2.5 h.

DISCUSSION

Although fibroblasts contain a low-potential cytochrome *b*-558 which is spectroscopically similar to the cytochrome *b*-558 of neutrophils [12], our current studies demonstrate that these proteins are structurally distinct. None of the six anti-(human neutrophil cytochrome *b*-558) antibodies tested by Western-blotting analysis showed a positive reaction with fibroblast membranes at concentrations of up to 1 mg of protein/lane, whereas neutrophil membranes showed characteristic positive reactions at concentrations of 5 µg of protein/lane. Since fibroblasts contain the low-potential *b*-558 in a concentration that is 10 % of that observed in neutrophils, a positive reaction should have been observed with fibroblast membranes at 50 µg of protein per lane. Not only the monoclonal antibodies, directed against distinct epitopes, but also the polyclonal antibodies, which recognize several epitopes on the cytochrome *b*-558 of neutrophils, showed no reaction with fibroblast membranes.

Recent studies by Radeke et al. [26] analysing fibroblast membranes using slot-blotting techniques with antibody 7D5 suggested that fibroblasts contained a cytochrome *b* with epitopes similar to human neutrophil protein. However, because this study utilized a slot-blot method to analyse fibroblast membranes, their conclusions are unsupported. Specifically, use of the slot-blot technique for analysing the presence or absence

of protein epitopes in crude samples does not control for the presence of other proteins that can non-specifically bind antibody. Indeed, our analysis of fibroblast membranes using standard Western-blotting techniques with antibody 7D5 indicated that 7D5 did not stain any bands in the region of the small subunit of the cytochrome, but rather some higher molecular-mass bands (80–85 kDa) unrelated to the cytochrome were stained. Clearly, this antibody did not recognize a cytochrome *b*-558 molecule in fibroblast membranes using Western blotting, but a slot-blot cannot make this distinction. In addition, it is not clear in the report by Radeke et al. [26] why SDS (12 %, w/v) abolished the binding of antibody 7D5 to cytochrome *b*-558 in PMNs, mesangial cells and fibroblasts, whereas Nakamura et al. [19], in the original report describing the properties of this antibody, observed a reaction of antibody 7D5 with PMN membranes even after treatment with 20 % SDS. This discrepancy further calls into question the specificity of the reactions observed by Radeke et al. [26] in fibroblasts.

Further support for our conclusion that the low-potential *b*-type cytochromes of phagocytes and fibroblasts are distinct protein species comes from our comparison of fibroblasts and PMNs from an X-linked CGD patient. Whereas the PMNs showed a decreased O_2^- release and cytochrome *b*-558 content compared with normal healthy donors, the fibroblasts of this patient showed no differences in the O_2^- -production rate or in cytochrome *b*-558 content compared with controls. In this form of CGD, the mutation is in the protein-coding region of the gene and not in the promoter or regulator gene. Thus, if both proteins were identical, fibroblasts and neutrophils should show the same protein defect and absence or decrease in cytochrome content, which is not the case. Thus these results provide direct genetic evidence that these two cytochrome species are indeed distinct. This conclusion is also supported by the results of Parkos et al. [29], who showed that mRNA of gp91-phox was absent from cells not belonging to the leucocyte line.

The use of fibroblasts derived from amniocentesis for prenatal detection of CGD has been suggested by Fikrig et al. [30], who observed differences in the reduction of NBT by fibroblasts stimulated with latex particles. However, these results could not be reproduced by other groups [31,32] who observed no differences in the reduction rate of NBT by stimulated CGD and normal fibroblasts. The quantitative determination of O_2^- production in fibroblasts of healthy donors and the CGD patient tested in this paper shows that the two are indistinguishable. Moreover, the cytochrome *b*-558 content in fibroblasts from the CGD patient is not decreased compared with fibroblasts of healthy donors. For these reasons, a prenatal diagnosis of CGD by cultured fibroblasts from amniocentesis is not possible.

The fact that the cytochromes in fibroblasts and neutrophils are different proteins with different genes is in accordance with the different regulation of the two systems. Some potent stimulants for neutrophils cause no or only a minor stimulation in fibroblasts, whereas stimulants triggering a high O_2^- release in fibroblasts lead only to a minor release of O_2^- in PMNs or do not stimulate these cells at all [33]. Moreover in contrast with PMNs, protein kinase C, tyrosine kinase and guanylate cyclase are not involved in the regulation of the fibroblastic O_2^- -generating system (B. Meier, A. J. Jesaitis, A. Emmendorffer, J. Roesler and M. T. Quinn, unpublished work). The use of different systems in phagocytes and mesenchymal cells is physiologically useful because of the different roles of the two systems. Whereas the NADPH oxidase system in phagocytes has an essential role in defence against infectious agents, it has regulatory functions in fibroblasts. Since activation of the neutrophil system not only leads to the killing of infectious

agents but also damages the neutrophils and surrounding tissues [34–36], an identical system in fibroblasts activated by the same compounds and mechanisms would be dangerous. During the evolutionary process, the phagocytic system appears to have developed a large number of components required for its activation [37,38], possibly in order to provide several levels of control necessary to prevent inappropriate activation of the NADPH oxidase which would also damage the host. On the other hand, the NADPH oxidase system in cells not involved in the defence system (e.g. fibroblasts) releases only low amounts of $O_2^{\cdot-}$ radicals, which are not damaging to these cells or surrounding tissue, to modulate cellular signals. In this case, such a complicated control mechanism is not as necessary as for the phagocyte system.

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