Restitution of Superoxide Generation in Autosomal Cytochrome-negative Chronic Granulomatous Disease (A22° CGD)-derived B Lymphocyte Cell Lines by Transfection with p22phox cDNA

By Friedrich E. Maly,* Cornelia C. Schuerer-Maly,+ Lawrence Quilliam,§ Charles G. Cochrane,* Peter E. Newburger,‖ John T. Curnutte,¹ Mary Gifford,‘‘ and Mary C. Dinauer”

From the *Department of Immunology, The Scripps Research Institute, La Jolla, California 92037; the †Department of Medicine, University of California at San Diego, San Diego, California 92093; the §Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599; the ‡Department of Pediatrics, University of Massachusetts, Worcester, Massachusetts 01655; the ††Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037; and *The Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana 46202

Summary

The respiratory burst oxidase of phagocytes and B lymphocytes is a multicomponent enzyme that catalyzes the one-electron reduction of oxygen by NADPH. It is responsible for the \( \text{O}_2^- \) production that occurs when these cells are exposed to phorbol 12-myristate 13-acetate or phagocytosis, or to cross-linking of surface immunoglobulin in B lymphocytes. The activity of this enzyme is greatly diminished or absent in patients with chronic granulomatous disease (CGD), an inherited disorder characterized by a severe defect in host defense against bacteria and fungi. In every CGD patient studied so far, an abnormality has been found in a gene encoding one of the four components of the respiratory burst oxidase: the membrane proteins p22phox or gp91phox which together form the cytochrome b558 protein, or the cytosolic proteins p47phox or p67phox. Autosomal recessive cytochrome-negative CGD (A22° CGD) is associated with mutations in the gene coding for p22phox. We report here that the capacity for \( \text{O}_2^- \) production and cytochrome b558 protein expression were restored to Epstein-Barr virus-transformed B lymphocytes from two A22° CGD patients by transfection with an expression plasmid containing a p22phox cDNA. No detectable \( \text{O}_2^- \) was generated by untransfected p22phox-deficient lymphocytes. The genetic reconstitution of the respiratory burst in A22° CGD B lymphocytes by transfer of the wild-type p22phox cDNA represents a further step towards somatic gene therapy for this subgroup of A22° CGD. This system will also be useful for expression of genetically engineered mutant p22phox proteins in intact cells, facilitating the structure-function analysis of cytochrome b558.

The \( \text{O}_2^- \)-forming NADPH oxidase of phagocytes (1) and B lymphocytes (2-4), also called the respiratory burst oxidase, is a multicomponent, membrane-bound enzyme that catalyzes the one-electron reduction of oxygen to \( \text{O}_2^- \) (1):

\[
2 \text{O}_2 + \text{NADPH} \rightarrow 2 \text{O}_2^- + \text{NADP}^+ + \text{H}^+.
\]

In resting phagocytes and B lymphocytes, the enzyme is not active, but acquires catalytic activity when the cells are stimulated by appropriate agents such as the protein kinase C activator PMA, or by phagocytosis (1) in case of phagocytes, and by cross-linking of surface Ig in B lymphocytes (3).

The respiratory burst oxidase plays a critical role in the generation of a complex group of reactive oxidants, including free radicals and oxidized halogens. In phagocytes, these oxidants serve as potent microbicidal agents (5). The role of \( \text{O}_2^- \) formation in B lymphocytes (2-4) which are non-phagocytic, noncytotoxic cells, is not yet defined. Oxidase activity in B lymphocytes is about 10% of that seen in phagocytes, as determined by \( \text{O}_2^- \) generation and by complementation assays in a cell-free oxidase-activating system (2-4, 6).

In the resting cell, oxidase components are distributed between the cytosol and the plasma membrane, but upon activation, the cytosolic components move to the plasma membrane to assemble the active enzyme (7, 8). Oxidase components that are always found in the membrane include a
respiratory burst oxidase–specific cytochrome, cytochrome b558, and an associated Ras-related low molecular weight G protein, rap1A (also designated Krev-1) (9–11). The cytochrome, postulated to be the terminal electron carrier in the electron pathway from NADPH to oxygen, is an oligomeric heme- and flavin-containing glycoprotein composed of the polypeptides p22phox and gp91phox (9, 12–14). rap1A has been postulated as an oxidative component since it copurifies with cytochrome b558 (11), however, its function within the oxidase complex has not yet been elucidated. The cytosolic oxidative components include the proteins p47phox and p67phox, both of which have been unequivocally established as essential elements of the oxidase (15) and one or both of the cytosolic GTP-binding proteins rac1 or rac2, each of which has been reported to support oxidative activity in a cell-free system (16, 17). During the cell activation process, all of these proteins migrate from the cytoplasm to the membrane.

Defective activity of the respiratory burst oxidase is the cause of an inherited disorder known as chronic granulomatous disease (CGD) (18). In this condition, phagocytes produce little or no O2−, and as a consequence, affected patients suffer from recurrent life-threatening infections. In all CGD patients studied to date, the abnormality in oxidative activity has been attributed to a defect in one of the four components: the α (p22phox) or β (gp91phox) subunit of cytochrome b558, p47phox, or p67phox. All of these oxidative components have been cloned (19–22). However, only the cytosolic proteins p47phox and p67phox have so far been expressed as functional proteins in bacterial fusion constructs and in the baculovirus expression system (21, 23, 24). Recombinant p47phox and p67phox have been shown to restore oxidative activity to p47phox− or p67phox−deficient cytoplasm in the cell-free, oxidative-activating system (23, 24), and transfection of an expression vector directing p47phox synthesis has been shown to restore oxidative activity to p47phox-deficient B lymphocyte lines (25, 26, 27).

Partially purified human neutrophil cytochrome b558 has been reported to reconstitute the defective oxidative activity of cytochrome-deficient CGD neutrophils in a cell-free oxidative activation system (28). However, attempts at expression of recombinant cytochrome b558 or its subunits in COS cells (29) and baculovirus expression systems (30) have met with limited success, and functionally active, recombinant cytochrome has thus far not been obtained. Cytochrome b558 is a structurally unusual cytochrome and shares only modest partial homology with other cytochromes. Furthermore, in CGD patients with mutations in one cytochrome subunit, the level of the unaffected subunit protein is generally greatly reduced (20, 31), suggesting that the single subunits are not stable in the absence of heterodimer formation. Mutations of the p22phox gene have been identified in patients with autosomal, cytochrome-negative (A22°) CGD (29), and mutations of the gene for gp91phox are found in patients with X-linked cytochrome-negative (X91°) CGD (19). However, the causal nature of the genetic defects in these subgroups of CGD has not been shown formally because of a lack of an expression system for generation of functionally active recombinant p22phox and gp91phox. Structure-function studies of the cytochrome b558 have also been hampered by the lack of a suitable system for expression of recombinant derivatives of p22phox and gp91phox, as altered by site-directed mutagenesis.

In this paper, we report the reconstitution of oxidative activity in intact autosomal cytochrome–negative CGD (A22° CGD)-derived B lymphocyte cell lines by stable transfection with an expression vector containing a cDNA that encodes p22phox.

Materials and Methods

Autosomal Cytochrome-negative CGD (A22° CGD) Patients. CGD patients were diagnosed as autosomal cytochrome-negative (A22° CGD) according to established clinical and laboratory criteria (32). The p22 gene of patient A22°:1 was sequenced and exhibits a nucleotide change (C-382→A) predicting a nonconservative amino acid change (Ser-118→Arg) in the mature p22phox protein (29). Patient A22°:2 was also characterized previously as autosomal recessive cytochrome negative CGD (33), and is unrelated to patient A22°:1.

Cell Lines and Medium. EBV-transformed B cell lines from A22° CGD patients were established by transformation of PBMC with EBV. Briefly, after obtaining informed consent, mononuclear cells were isolated from samples of venous blood and cultured with supernatant of the B95-8 marmoset B cell line containing EBV virions, using RPMI 1640 medium supplemented with 20% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), the antibiotics penicillin, streptomycin, and fungizone, and buffered to pH 7.2. Cells were kept in a humidified incubator at 37°C gassed with ambient air plus 5% CO2. Continuously growing EBV-transformed cell lines were obtained after 5–6 wk and were carried further in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, the antibiotics penicillin, streptomycin, and ciprofloxacin, and buffered with 20 mM Hepes to pH 7.2 (3). Viability of EBV-transformed cell lines was routinely >90% by trypan blue exclusion test. EBV-transformed B lymphoblast lines from patients with X-linked CGD (X91°-CGD B cell lines) and from normal individuals (wild-type EBV B cell lines) were derived in an analogous fashion.

The human PLB-985 myelomonoblastic cell line has been described earlier (34). To obtain granulocyte-like forms, PLB-985 cells were exposed to 60 mM dimethylformamide for 6 d. The levels of cytochrome b558 in these induced cells is about 50% of that seen in normal peripheral blood neutrophils (our unpublished observation).

Construction of Expression Vector pEBoP22phox. The expression vector pEBoP22phox was constructed by inserting a full-length p22phox cDNA into the episomal expression vector pEBoLPp, pEBoLPp, a 10.6-kb plasmid constructed from portions of the four plasmids pCD, pl, pUC19, and EBoPSVNeo (35) was from R. Margolskee (Roche Research Institute, Nutley, NJ) and F. V. Chisari (The Scripps Research Institute). The vector contains the following functional units: a pBR322 origin, a β-lactamase-gene, an EBNA-1 gene driven by a SV40 late promoter, a gene encoding hygromycin phosphotransferase driven by an SV40 early promoter, an SV40 late-region intervening sequence, the SV40 origin of replication oriP, and a polylinker region containing unique sites 5′-SacI-HindIII-

1 Abbreviations used in this paper: CGD, chronic granulomatous disease; NBT, nitroblue tetrazolium.
XbaI-Sal-NotI-KpnI-3' the insert of which is driven by a second SV40 early promoter. A full-length clone of p22phox cDNA (20) was subcloned into the EcoRI site of pcEM9 (Promega, Madison, WI). The 700-bp insert containing p22phox cDNA was then directionally cloned in the sense orientation into the HindIII-SstI site of pH2LOLP. The final construct, pEBOp22phox, was amplified in Escherichia coli DH5α and purified by alkaline lysis and cesium chloride banding for transfection of cell lines. Where not otherwise indicated, molecular genetic manipulations were performed as described by Sambrook et al. (36).

Transfection and Selection. pEBOp22phox plasmid DNA was introduced into A22° CGD lymphocyte cell lines by electroporation (37). For this, cell lines were kept in logarithmic growth phase by splitting them 1:2 for three consecutive days. Before electroporation, cells were transferred into serum-free RPMI 1640 medium supplemented with 1 mM glucose and 0.1 mM dithiothreitol to give a final density of 20 × 10⁶/ml. 500 µl cell suspension (10⁷ cells) were mixed gently with 10 µg plasmid DNA in an electroporation cuvette (0.4 cm electrode distance, BioRad Laboratories, Cambridge, MA) and immediately subjected to electroporation at 200 V and 960 µF using a gene pulser (BioRad Laboratories). These manipulations were done at ambient temperature under sterile conditions. Transfection efficiency was about 5% using β-galactosidase as reporter gene. Immediately after the electroporation pulse, cells were transferred to 10 ml of complete culture medium. After 48 h of culture, selection of transfectedants started by adding hemoglobin at 50 µg/ml. Selection of transfectedants was complete after 4 wk of culture in hemoglobin; selected cells were maintained at 25 µg/ml hemoglobin Viability of transfected lines was 80-90% as assessed by trypan blue exclusion.

Isolation and Analysis of RNA. Total RNA was isolated from B cell lines by the acid guanidinium thiocyanate method (38). Northern blot analysis with 32P-labeled cDNA probes was performed essentially as previously described (29). Briefly, samples were electrophoresed on denaturing formaldehyde-agarose gels, transferred to nylon membranes (Magnagraph; Micron Separations, Westboro, MA), hybridized with radiolabeled probes using conditions suggested by the manufacturer, and washed under high stringency conditions. The p22phox probe was a full-length cDNA (20). The human β-actin cDNA was obtained from C. Srivastava (Indiana State University School of Medicine, Indianapolis, IN).

Immunoblot Analysis of Protein Expression. Triton X-100 extracts of whole cells (19) and of cytosolic and membrane fractions isolated from sonicated cells (8) were prepared for immunoblot analysis as described (19). Antibody binding was detected using a chemiluminescent method using a horseradish peroxidase-conjugated second Ab (Amersham Corp., Arlington Heights, IL). The p22phox Ab was affinity purified from rabbits immunized with a synthetic peptide derived from p22phox sequence (39). The gp91phox Ab was a mouse mAb raised to purified cytochrome b (31), kindly provided by Dr. Dirk Roos (The Netherlands Red Cross Central Laboratory, Amsterdam, The Netherlands).

Chemiluminescence Monitoring of Peroxide Generation by B Cells. Cells were transferred from culture medium into phenol red-free MEM (GIBCO BRL, Gaithersburg, MD), supplemented with BSA (100 µg/ml) and buffered with 5 mM Hepes, pH 7.2, to give a final cell density of 5 × 10⁶/ml. Luminol (100 µM final concentration) and horseradish peroxidase (10 U/ml) were added for luminescence detection of hydrogen peroxide (40). A 200-µl cell suspension, containing 10⁶ cells, was placed into the wells of a white opaque microtiter plate (Dynatech Laboratories Inc., Chantilly, VA). After adding stimuli, chemiluminescence was recorded as 4-h integrals or kinetically by repeated recordings of 15- or 30-min integrals by single photon imaging in a camera luminometer (Argus 100; Hamamatsu Photonic Systems Corp., Bridgewater, NJ) thermostatted to 37°C as described earlier (41).

Nitroblue Tetrazolium (NBT) Reduction. NBT reduction was used to estimate the frequency of superoxide-generating cells as described earlier (4). Briefly, cells at a density of 2 × 10⁶/ml in RPMI 1640 medium containing 10% FCS were stimulated at 37°C with PMA (100 ng/ml) in the presence of 1 mg/ml NBT.

The reaction was stopped by the addition of an equal volume of ice-cold 2% paraformaldehyde and the frequency of NBT-positive (blue) cells determined by light microscopy.

Reagents. NBT, Luminol, glucose, dithiothreitol, PMA, horseradish peroxidase, and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO). Protein A-bearing staphylococi (Pansorbin) and hygromycin were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Results

Northern Blot Analysis of p22phox. To verify the effectiveness of transcription from the plasmid pEBOp22phox transfected into B cell lines, Northern blots were performed using a p22phox probe. Normal wild-type EBV B cells contained p22phox mRNA of the expected size (0.9 kb), which was barely detectable in both A22° CGD-derived EBV B cell lines. In contrast, after transfection with pEBOp22phox, both A22° CGD-derived EBV B cell lines contained an abundant 1.6-kb plasmid-derived p22phox mRNA species in addition to the endogenous 0.9-kb transcript (Fig. 1).

Immunoblot Analysis of Cytochrome b558 Subunit Proteins. Next, cellular expression of the p22phox cytochrome subunit was investigated by immunoblotting. Granulocyte-induced PLB-985 cells, wild-type EBV B cell lines and, to a much lesser extent, X91° CGD-derived EBV B cell lines expressed p22phox immunoreactive protein. In contrast, p22phox immunoreactive protein was undetectable in both parental A22° CGD lines. However, correlating with the abundant production of plasmid-derived p22phox mRNA, A22° CGD lines transfected with EBO-p22phox contained readily detectable p22phox protein, at levels equal to or even exceeding those seen in wild-type EBV B cell lines (Fig. 2, top). The p22phox in the wild-type B cell line and the A22° line transfected with EBO-p22phox was localized to the membrane fraction (Fig. 2, top), as expected.

It has been noted previously that A22° CGD neutrophils also lack the glycosylated large cytochrome subunit, gp91phox, apparently because of a lack of stabilizing interaction with p22phox (29, 42). It was therefore of interest to determine expression of gp91phox in A22° CGD-derived B cell lines before and after correction of p22phox expression by transfection. Using a mAb raised to purified cytochrome b (31), the gp91phox detected in wild-type B cell lines appeared as a diffuse band ranging from about 55 to 95 kD, in contrast to its appearance as a band centered at 90-110 kD in granulocyte-induced PLB-985 cells (Fig. 2, bottom) and peripheral blood neutrophils (19). This finding may reflect differences in glycosylation between B cells and myeloid cells. A22° CGD-derived B cell lines contained a membrane-
associated immunoreactive protein of about 55 kD, which was absent in an X91° B cell line, that may represent un-glycosylated gp91 phox (Fig. 2, bottom). After transfection with EBO-p22 phox, both A22° CGD–derived B cell lines contained forms of gp91 phox ranging from about 55 to 100 kD, now resembling the wild-type B cells (Fig. 2, bottom). All of the EBV B cell lines examined had a prominent band of about 100 Kd (Fig. 2, bottom), which localized to the cytosolic fraction in sonicated cells. As this species was also present in a cell line derived from a patient with X91° CGD (Fig. 2, bottom), it is likely to represent a cross-reactive protein.

Transfection with pEBOp22 phox Restores Oxidase Activity. Before transfection with p22 phox, both A22° CGD–derived B cell lines failed to generate chemiluminescence and to reduce NBT in response to stimulation with either phorbol ester PMA or the surface Ig cross-linking reagent, protein A (Table 1 and Fig. 3). This inability to perform an oxidative burst is in line with previous reports on the missing oxidase activity of CGD-derived, EBV-transformed B cell lines (2). In contrast, the transfectants A22°:1/pEBOp22 and A22°:2/pEBOp22 clearly possessed oxidase activity (Fig. 3 and Table 1). This was evident both in the NBT reduction assay which is sensitive to superoxide, and in the chemiluminescence assay which detects hydrogen peroxide. The frequency of NBT-positive cells among PMA-stimulated A22°:2/pEBOp22 was about double that of PMA-stimulated A22°:1/pEBOp22 cells, and in parallel, A22°:2/pEBOp22 cells exhibited higher chemiluminescence than A22°:1/pEBOp22. Interestingly, in addition to phorbol ester PMA, pEBOp22-transfected A22° CGD–derived B cell lines also responded with oxidase activity in response to cross-linking of surface Ig by protein A (Fig. 3) which represents a more physiologic and receptor-mediated stimulus of NADPH oxidase in normal EBV B cell lines (3). The relative level of oxidase activity of the A22° pEBOp22 transfectants (as measured both by NBT reduction and chemiluminescence) was within the range of oxidase activity seen in a panel of normal...
Table 1. NBT Reduction by A22° CGD B Cell Lines before and after Transfection with pEBOp22™

| Cell line     | Additions | Incubation time | NBT-positive cells |
|---------------|-----------|----------------|-------------------|
| A22°:1        | —         | 30             | 0.2*              |
|               |           | 60             | 1.06              |
| A22°:1/pEBOp22| —         | 30             | 1.75              |
|               | —         | 60             | 8.08              |
|               | SOD       | 60             | 2.2               |
|               | Boiled SOD | 60          | 7.94              |
|               | HSA       | 60             | 8.2               |
| A22°:2        | —         | 30             | 0.3               |
|               |           | 60             | 2.5               |
| A22°:2/pEBOp22| —         | 30             | 3.9               |
|               |           | 60             | 23.1              |
|               | SOD       | 60             | 3.2               |
|               | Boiled SOD | 60          | 21.2              |
|               | HSA       | 60             | 22.2              |

* 10⁶ cells in 500 µl RPMI/10% FCS with 1 mg/ml NBT were stimulated with 100 ng/ml PMA and incubated at 37°C. The reaction was stopped with ice-cold 2% paraformaldehyde/PBS and the frequency of NBT-positive (blue) cells determined by light microscopy from a minimum of 500 cells counted.

† Superoxide dismutase, 100 µg/ml.
§ SOD boiled for 15 min, 100 µg/ml.
¶ Human serum albumin, 100 µg/ml.

EBV B cell lines (data not shown). Transfection of wild-type, oxidase-competent EBV B cell lines with pEBOp22™ had no effect on oxidase activity (data not shown).

Discussion

Mutations in the p22phox gene, which codes for the small subunit of cytochrome b558, have previously been identified in several patients with autosomal cytochrome-negative CGD (29). However, in the absence of an expression and assay system for natural or mutant cytochrome b558, it could not be formally demonstrated that the missense variants result in a defective p22phox cytochrome subunit. Here, we made use of the fact that the NADPH oxidase of phagocytes is also present in B lymphocytes (2-4) and that EBV-transformed B lymphocyte cell lines generated from CGD patients share the genetic defects apparent in the patients' phagocytes (2). Transfection of A22° CGD-derived B cell lines with an expression vector containing a wild-type p22phox cDNA led to production of recombinant p22phox protein, "rescue" of the large subunit of cytochrome b558, and most importantly, to restoration of the capacity to generate an oxidative burst in response to defined stimuli. Restitution of p22phox expression and oxidase activity was observed in cell lines derived from two unrelated A22° CGD patients. These data represent the first formal demonstration that the respiratory burst in CGD patients with p22phox gene defects can be reconstituted by expression of wild-type p22phox. These results also underscore the importance of cytochrome b558 expression for intact oxidase function. We (25) and other authors (26, 27) have previously used similar approaches to functionally correct genetic defects in EBV-transformed B cell lines from p47phox-deficient patients. The ability to achieve genetic
reconstitution of CGD B cell lines is a useful in vitro model in which to develop an experimental framework for somatic gene therapy of this life-threatening disease.

Although the cDNAs for the p22<sub>phox</sub> and gp91<sub>phox</sub> subunits of cytochrome b558 have been isolated some years ago (19, 20), the functional organization of this heterodimer has been largely uncharacterized. The p22<sub>phox</sub>-deficient B cell lines derived from A22<sup>°</sup> CGD patients provide a useful whole cell model for expression of recombinant p22<sub>phox</sub> and for evaluation of the effects of specific modifications in p22<sub>phox</sub> as introduced by site-directed mutagenesis. Superoxide and derived reactive oxygen species not only serve useful microbicidal functions, but can also cause serious tissue damage. The excessive generation of phagocyte-derived oxidants has been implicated in the pathogenesis of a wide variety of inflammatory conditions (5, 43). Definition of critical functional domains within cytochrome b558 may aid in the rational design of antiinflammatory agents that specifically interfere with superoxide formation.

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Address correspondence to Dr. F. E. Maly, Institute of Physiology, University of Zurich, Winterthurer Strasse 190, CH-8057 Zurich, Switzerland.

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