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O_2^- production by B lymphocytes lacking the respiratory burst oxidase subunit $p47^{phox}$ after transfection with an expression vector containing a $p47^{phox}$ cDNA

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ABSTRACT **The respiratory burst oxidase of phagocytes and B lymphocytes is a complicated enzyme that catalyzes the one-electron reduction of oxygen by NADPH. It is responsible for the O_2^- production that occurs when these cells are exposed to phorbol 12-myristate 13-acetate or other appropriate stimuli. The activity of this enzyme is greatly decreased or absent in patients with chronic granulomatous disease, an inherited disorder characterized by a severe defect in host defense against bacteria and fungi. In every chronic granulomatous disease patient studied to date, an abnormality has been found in a gene encoding one of four components of the respiratory burst oxidase: the membrane protein $p22^{phox}$ or $gp91^{phox}$, or the cytosolic protein $p47^{phox}$ or $p67^{phox}$. We report here that O_2^- production was partly restored to phorbol 12-myristate 13-acetate-stimulated Epstein–Barr virus-transformed B lymphocytes from a patient with $p47^{phox}$-deficient chronic granulomatous disease by transfection with an expression plasmid containing a $p47^{phox}$ cDNA inserted in the sense direction. No detectable O_2^- was produced by untransfected $p47^{phox}$-deficient lymphocytes or by $p47^{phox}$-deficient lymphocytes transfected with an antisense plasmid. The finding that O_2^- can be produced by $p47^{phox}$-deficient B lymphocytes after the transfer of a $p47^{phox}$ cDNA into the deficient cells suggests that this system could be useful for studying the function of mutant $p47^{phox}$ proteins in whole cells.**

The O_2^--forming NADPH oxidase of phagocytes and B lymphocytes (the respiratory burst oxidase) is a multicomponent membrane-bound enzyme that catalyzes the one-electron reduction of oxygen to O_2^- (1):

$$2\ O_2 + NADPH \rightarrow 2\ O_2^- + NADP^+ + H^+$$

The enzyme is dormant in resting phagocytes but acquires catalytic activity when the cells are stimulated by any of a wide variety of agents.

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 (2). The O_2^--forming oxidase is also found in B lymphocytes (3, 4), where its function is not clear because these lymphocytes do not themselves possess microbicidal activity. Oxidase activity in B lymphocytes is ≈10% that seen in phagocytes, as determined by O_2^- generation and by complementation assays in a cell-free oxidase-activating system (3–5).

In the resting cell the components of the oxidase are distributed between the cytosol and the plasma membrane, but on activation the cytosolic components move to the plasma membrane to assemble the active enzyme (6, 7). The components of the oxidase that are always found in the membrane include a phagocyte-specific cytochrome—cytochrome b_{558}—and possibly an associated G protein, rap 1A (also designated Krev-1) (8–10). The cytochrome, postulated to be the terminal electron carrier in the electron-transport pathway between NADPH and oxygen, is an oligomeric heme- and flavin-containing glycoprotein composed of the polypeptides $p22^{phox}$ and $gp91^{phox}$ (8, 11–13). Rap 1A has been proposed as an oxidase component because it copurifies with cytochrome b_{558} (10).

The cytosolic components include the proteins $p47^{phox}$ and $p67^{phox}$, both of which have been unequivocally established as essential elements of the oxidase (14) and one or more of the cytosolic GTP-binding proteins rac 1 or rac 2, each of which has been reported to support oxidase activity in a cell-free system (15–17). All of these proteins migrate from the cytoplasm to the membrane during the activation process. A 26-kDa cytosolic GDP dissociation inhibitor known as GDI may also be involved in the regulation of oxidase activity because this protein has been shown to associate with rac 1 (16).

A defect in the activity of the respiratory burst oxidase is the cause of an inherited disorder known as chronic granulomatous disease (18). In this condition, phagocytes make little or no O_2^-, and as a consequence, affected patients suffer from recurrent life-threatening infections because of a failure of microbicidal oxidant production. In all chronic granulomatous disease patients studied so far, the abnormality in oxidase activity has been attributed to a defect in one of four components: the α ($p22^{phox}$) or β ($gp91^{phox}$) subunit of cytochrome b_{558}, $p47^{phox}$, or $p67^{phox}$. All of these oxidase components have been cloned (19–23), and the cytosolic proteins $p47^{phox}$ and $p67^{phox}$ have been expressed in bacterial-fusion constructions (21, 23), in the baculovirus expression system (24), and transiently in COS cells (S.J.C., unpublished work). At present, however, the only system available for studying the function of these cytosolic proteins is a cell-free oxidase-activating system that appears to model only the last step or two of the signal-transduction pathways responsible for oxidase activation *in vivo* (25–28). To obtain a more complete picture of the function of these proteins, it would be desirable to develop an experimental system in which the entire signal-transduction pathway from receptor to enzyme is intact. As a step in this direction, we report the reconstitution of oxidase activity in intact $p47^{phox}$-deficient B lymphocytes by stable transfection with an expression vector containing the cDNA that encodes $p47^{phox}$.

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Abbreviations: SV40, simian virus 40; PMA, phorbol 12-myristate 13-acetate; EBV, Epstein–Barr virus.

MATERIALS AND METHODS

Construction of Expression Vectors pEBOp47fx$^+$ and pEBOp47fx$^-$. These expression vectors were constructed by inserting the p47^{phox} cDNA into the expression plasmid EBOpLPP, a shuttle vector designed to express genes in Epstein–Barr virus (EBV)-transformed B lymphocytes. This vector, a 10.6-kilobase (kb) plasmid constructed from portions of the four plasmids pcDV, pL, pUC19, and EBOpSV2neo (29) was from R. Margolskee (Roche Institute, Nutley, NJ) and F. V. Chisari (Scripps Research Institute, La Jolla, CA). The vector contains the following functional units: a pBR322 origin, a β-lactamase gene, an *EBNA-1* gene driven by a simian virus 40 (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′-*Sac* I–*Hin*dIII–*Xba* I–*Sal* I–*Not* I–*Kpn* I-3′ the insert of which is driven by a second SV40 early promoter. The full-length cDNA for p47^{phox} was obtained from a retinoic acid/dimethylformamide-differentiated HL-60 λgt10 cDNA library (from Stuart H. Orkin, Children's Hospital Medical Center, Boston) by amplification with the PCR using the unique primers ATGGGGGACACCTTCATCCGTCAC, which corresponded to the 5′ end of the open reading frame, and CACTCCAAGCAACATTTATTG, which matched a sequence in the 3′-untranslated region downstream from the translation stop site. The 1.3-kb cDNA was first inserted into pBluescript KS+ (Stratagene). Directional cloning into plasmid EBOpLPP was then accomplished by excising the cDNA from Bluescript with an appropriate pair of enzymes and inserting it into EBOpLPP gapped with the same enzymes: *Xba* I to *Not* I for the sense direction and *Xba* I to *Hin*dIII for the antisense direction. Orientation was confirmed by double-stranded dideoxynucleotide chain-termination sequencing. The final expression plasmids, designated pEBOp47fx$^+$ and pEBOp47fx$^-$ respectively, were amplified in *Escherichia coli* DH5α and purified by alkaline lysis/CsCl. Where not otherwise indicated, molecular genetic manipulations were done as described by Sambrook *et al.* (30).

Transformation of Lymphocytes by EBV. Peripheral blood was obtained from a p47^{phox}-deficient patient and a normal subject after informed consent was given, according to procedures approved by institutional review boards. Mononuclear cells were isolated by Ficoll/Hypaque centrifugation (31) and either used immediately or frozen in 10% (vol/vol) dimethyl sulfoxide in a programmable cell freezer. Lymphoblastoid cell lines were initiated by incubation of 1–2 × 10^6 mononuclear cells with high-titer EBV supernatants from the B95-8 marmoset cell line as described (32, 33). Cultures were grown in RPMI 1640 medium/10% heat-inactivated fetal calf serum/penicillin/streptomycin/fungizone.

Transfection. For transfection, 2 × 10^7 lymphocytes were washed and then suspended in 0.30 ml of electroporation buffer (6 μM glucose/137 μM NaCl/5 μM KCl/20 μM Hepes/0.7 μM Na_2HPO_4, pH 7.04) containing 10 μg of plasmid DNA and 10 μg of SV40 DNA (Bethesda Research Laboratories). The suspension was placed in a Bio-Rad gene pulser cuvet with an electrode gap of 0.4 cm, incubated for 15 min at room temperature, and then exposed at room temperature to a pulse of direct current at 210 V using a Bio-Rad gene pulser (with extended capacitance) at a capacitance of 960 μF. Under these conditions, the cells were transfected at 5–10% efficiency. After an additional 15 min, the cell suspension was added to 3 ml of RPMI 1640 medium/10% (vol/vol) fetal calf serum/1% (vol/vol) antibiotic–antimycotic solution (Sigma)/2 mM glutamine and placed in an incubator at 37°C. After 3 days, the medium was replaced with the same medium containing hygromycin B at 150 μg/ml (Sigma). The cells were grown in hygromycin-containing medium until they reached numbers suitable for the experiments.

Immunoblotting. Immunoblotting was done by using a rabbit polyclonal antibody raised against the peptide CFSTKRKLASAV, which contains the C-terminal decapeptide of p47^{phox} (34). For each sample, 1.2 × 10^6 lymphocytes were lysed in detergent-containing buffer (0.15 M NaCl/50 mM Tris, pH 8/5 mM EDTA/1% Tween 20). Nuclei were removed at 4°C for 15 min at 2500 × *g*, and the lysates were clarified at 105,000 × *g* for 30 min. The supernatants were electrophoresed according to Laemmli (35) on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose as described by Towbin *et al.* (36). The resulting blot was preblocked with Blotto for 2 hr at room temperature, probed overnight with a 1:250,000 dilution of ammonium sulfate-precipitated anti-p47^{phox} antibody (10.4 mg/ml), then treated for 2 hr with alkaline phosphatase-coupled goat anti-rabbit IgG, and finally visualized using an alkaline phosphatase-conjugate substrate kit from Bio-Rad.

Assay of O_2^- Production (37). Luminometer cuvets contained 3 × 10^6 lymphocytes, lucigenin (final concentration, 0.1 mM), superoxide dismutase (final concentration, 100 μg/ml) where indicated, and enough minimum essential medium (MEM) to bring the volume to 0.45 ml. The MEM was free of serum and phenol red but contained 0.01% bovine serum albumin and 20 mM Hepes. The cuvets were wrapped with aluminum foil to exclude light and then warmed at 37°C for 5 min. Some samples were then activated with phorbol 12-myristate 13-acetate (PMA; 50 μl at 10 μg/ml in MEM/1% dimethyl sulfoxide); the remaining samples received buffer alone (50 μl of 1% dimethyl sulfoxide/MEM). After incubation in the dark for 15 more min at 37°C followed by 2 hr at room temperature, the samples were placed in a Monolight 2010 luminometer operating at room temperature, and chemiluminescence was determined over consecutive 30-s intervals. Light emission from assay mixtures containing PMA but no cells was 17.7 ± 0.4 counts per 30 s.

RESULTS

The small amounts of O_2^- produced by B lymphocytes can be detected by lucigenin, which emits light when it reacts with O_2^-. As earlier work has shown and the results of Table 1 confirm, stimulated but not resting B lymphocytes manufacture O_2^-, as indicated by the light emission from reaction mixtures containing normal B lymphocytes, PMA, and lucigenin. The O_2^- generated by these B lymphocytes is the product of the respiratory burst oxidase because no light was emitted from otherwise similar reaction mixtures containing PMA-treated p47^{phox}-deficient B lymphocytes.

Fig. 1 shows the results of experiments conducted with p47^{phox}-deficient B cells transfected with pEBOp47fx$^+$ and

Table 1. O_2^- production by normal and p47^{phox}-deficient B lymphocytes

Lymphocytes	Light emission, counts per 30 s	
	Exp. 1	Exp. 2
Normal		
Resting cells*	2.1 ± 0.7	−3.7 ± 0.8
Stimulated cells	2222.1 ± 15.2	1240.0 ± 53.6
p47^{phox}-deficient		
Resting cells	0.7 ± 0.7	1.4 ± 0.3
Stimulated cells	1.6 ± 1.0	0.3 ± 0.7

The experiments were done as described in the text. Values are the mean ± SD of counts from the first five 30-s intervals after placing cells in the luminometer, corrected for a blank value of 17.7 counts per 30 s.
*These two incubations each contained 2 × 10^6 lymphocytes.

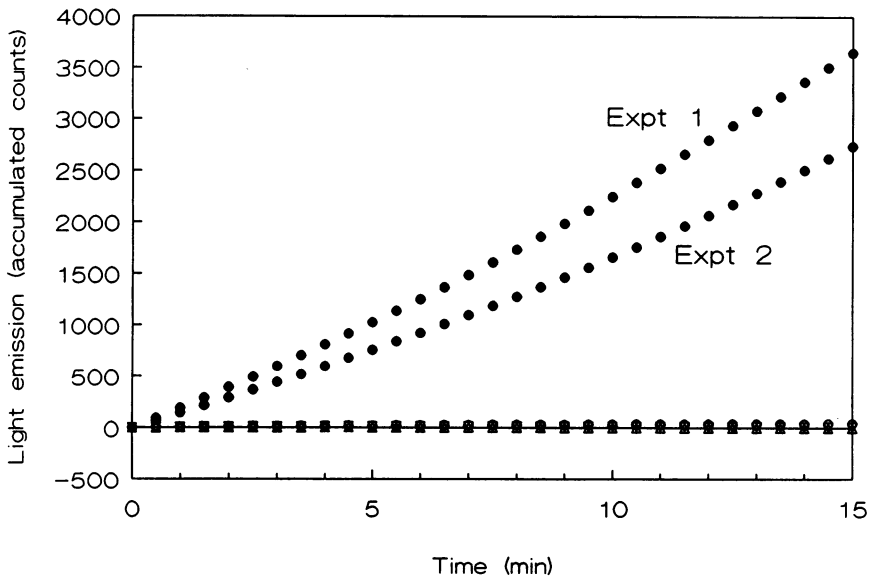

FIG. 1. O_2^- production by $p47^{phox}$-deficient lymphocytes transfected with pEBOp47fx$^+$ (sense) and pEBOp47fx$^-$ (antisense) constructs. Experiments were conducted as described in the text. ●, Sense, PMA; ○, sense, resting; ▽, antisense, PMA; △, antisense, resting. Exp. 1 and Exp. 2 represent individual assays of light emission by stimulated sense-transfected cells. Other results are the averages of duplicate assays that agreed within ± 10%.

pEBOp47fx$^-$, which contain $p47^{phox}$ cDNA inserts oriented to transcribe $p47^{phox}$ messages in the sense and antisense directions, respectively. No O_2^- was detected in reaction mixtures containing antisense-transfected B lymphocytes incubated either with or without PMA, nor was it produced in reaction mixtures containing sense-transfected B cells incubated in the absence of PMA. O_2^- was generated, however, when the sense-transfected lymphocytes were stimulated with PMA, as shown by the emission of light from reaction mixtures containing both sense-transfected cells and PMA. Light emission from those reaction mixtures was suppressed by superoxide dismutase (Table 2), confirming that O_2^- was the source of the luminescence.

Immunoblotting was used to identify the $p47^{phox}$ gene product in the $p47^{phox}$-deficient lymphocytes both before and after transfection. $p47^{phox}$ was readily demonstrated in both normal B lymphocytes and $p47^{phox}$-deficient B lymphocytes transfected with the sense plasmid pEBOp47fx$^+$ (Fig. 2) but could not be detected in untransfected or antisense-transfected $p47^{phox}$-deficient lymphocytes. The distribution of $p47^{phox}$ among these various lymphocyte lines is, therefore, in accord with the ability of the cells to generate O_2^- in response to a stimulus.

DISCUSSION

A potentially useful system for studying the respiratory burst oxidase would be one in which transfection with an appropriate cDNA restores O_2^--generating ability to cells lacking an oxidase component. In principle, phagocytes would be ideal for such a system because they manufacture large quantities of O_2^- in response to well-defined stimuli. At present, however, a myeloid line deficient in an oxidase component is unavailable. Even if such a line were available, success would be highly uncertain because of the many technical problems encountered in transfections of myeloid cells.

B lymphocytes, which are much more experimentally tractable than phagocytes, produce a burst of O_2^- when exposed to appropriate stimuli. As with phagocytes, O_2^- production by lymphocytes is greatly diminished or absent in chronic granulomatous disease, indicating that the enzyme responsible for O_2^- production in lymphocytes is probably identical to the respiratory burst oxidase of phagocytes (33, 38, 39). Because B lymphocytes are easier to transform and manipulate and because they express the respiratory burst oxidase, those cells rather than phagocytes were chosen as transfection targets. Cobbs *et al.* (40) reported $p47^{phox}$ biosynthesis but not O_2^- production by EBV-transformed $p47^{phox}$-deficient B lymphocytes transfected with a retrovirus

Table 2. Effect of superoxide dismutase on light emission from incubations containing sense-transfected $p47^{phox}$-deficient lymphocytes

Conditions	Light emission, counts per 30 s	
	Exp. 1	Exp. 2
Resting cells		
Without SOD	4.4 ± 1.0	
With SOD	2.8 ± 1.1	2.2 ± 0.6
Stimulated cells		
Without SOD	113.7 ± 1.6	94.5 ± 1.0
With SOD	3.7 ± 0.8	3.5 ± 1.1

The experiments were done as described in the text. Values are the mean ± SD of counts obtained during the first five 30-s intervals after placing the cells in the luminometer, corrected for a blank value of 17.7 counts per 30 s. SOD, superoxide dismutase.

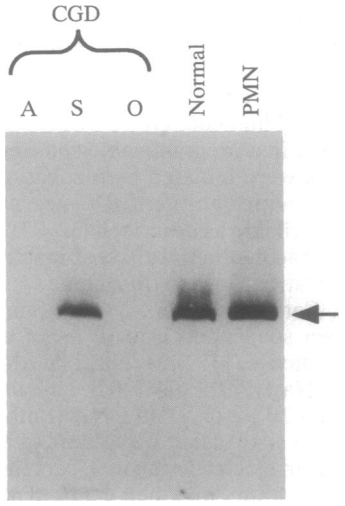

FIG. 2. Immunoblots of $p47^{phox}$ in normal lymphocytes, transfected and untransfected $p47^{phox}$-deficient B lymphocytes, and neutrophil cytosol. Immunoblotting was done as described. Lanes: A, EBV-transformed $p47^{phox}$-deficient B lymphocytes transfected with pEBOp47fx$^-$ (antisense); S, EBV-transformed $p47^{phox}$-deficient B lymphocytes transfected with pEBOp47fx$^+$ (sense); O, untransfected EBV-transformed $p47^{phox}$-deficient B lymphocytes; Normal, untransfected EBV-transformed normal B lymphocytes; PMN, neutrophil cytosol. Location of $p47^{phox}$ is indicated by arrow.

containing a $p47^{phox}$ cDNA insert. Using a different assay for O_2^- than the one used by Cobbs *et al.*, we observed both $p47^{phox}$ production and O_2^- generation by EBV-transformed $p47^{phox}$-deficient B lymphocytes stably transfected with the episomal plasmid pEBOp47fx$^+$, which was constructed from the expression vector EBOpLPP and a full-length $p47^{phox}$ cDNA. We believe that this system could be useful for examining the function of mutant $p47^{phox}$ proteins in whole cells.

The rate of O_2^- production by sense-transfected $p47^{phox}$-deficient lymphocytes as compared with the rate seen with normal B lymphocytes (Table 1) suggests that the respiratory burst in the transfected cells was restored to the extent of 5–10%; this comparison, however, may be misleading. We have found that O_2^- production by normal EBV-transformed B lymphocytes is highly variable, ranging in our experiments from <350 counts per 30 s to well over 10,000 counts per 30 s. These results suggest that the capacity of B lymphocytes for O_2^- production is subject to complex regulatory influences, the nature of which is presently obscure but which could be related to growth conditions, transfection, or other factors. For this reason we believe it is premature at this stage to try to interpret differences in the rates of O_2^- production between normal and transfected cells in terms of percent recovery of the respiratory burst. Similarly, the interpretation of quantitative differences in O_2^- production by cells transfected with "wild-type" vs. mutant plasmids is not straightforward, although a complete failure of O_2^- production by lymphocytes transfected with a pEBOp47fx$^+$ mutant can be interpreted in a clear-cut manner.

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