RAPID COMMUNICATION

Restoration of Superoxide Generation to a Chronic Granulomatous Disease-Derived B-Cell Line by Retrovirus Mediated Gene Transfer

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Failure of a superoxide generating system, the NADPH oxidase, present in neutrophils and other phagocytes gives rise to chronic granulomatous disease (CGD), a group of single-gene inherited disorders all characterized by an extreme susceptibility to pyogenic infection, with potentially fatal consequences. About 30% of CGD cases are caused by an autosomally inherited deficiency of a 47-Kd cytoplasmic component of the oxidase (p47-phox). Epstein-Barr virus (EBV) immortalized B-lymphocyte lines established from these CGD patients also express this NADPH oxidase defect and consequently are rendered incapable of generating superoxide on stimulation. We have used a p47-phox-deficient EBV-transformed B-cell line as a recipient for retroviral transfer of a functional p47-phox cDNA. The presence and activity of the retrovirally encoded p47-phox in the transduced cells is demonstrated and we show that this restores their capacity to generate superoxide.

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MATERIALS AND METHODS

Retroviral vector construction and establishment of producer cell line. A p47-phox cDNA lacking a poly A addition signal was constructed from a plasmid containing a full-length cDNA10 by cleaving at the NheI site approximately 100 nucleotides (nt) upstream, and reclosing the plasmid at the compatible XhoI site located in the plasmid multiple cloning site. The cDNA containing fragment was excised with EcoRI and SalI, gel purified and cloned into the corresponding sites of pPBabe Neo.20 The pBabe Neo 47Δ DNA (10 μg) was introduced into the PA31721 packaging cell line by standard calcium phosphate transfection. Individually arising G418 (1 mg/mL) resistant clones were isolated by ring cloning and titered for their production of recombinant retrovirus by colony assay on NIH 3T3 cells. The clone producing the highest titer retrovirus (6 × 10^6 cfu/mL) was used to infect a p47-phox-deficient B-cell line by cocultivation. After continuous growth in selection (G418 2 mg/mL) for 6 weeks, cells were assayed for their ability to produce superoxide.

Superoxide assays. For luminometry, B cells were harvested, washed twice in phosphate-buffered saline (PBS) (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na2HPO4, pH 7.4), and resuspended in Hank's buffered saline solution (HBSS) with calcium and magnesium (137 mmol/L NaCl, 5.4 mmol/L KCl, 358 mmol/L NaHCO3, 0.44 mmol/L KH2PO4, 0.34 mmol/L Na2HPO4, 0.5 mmol/L CaCl2, 1 mmol/L MgCl2) in the presence of 10 μmol/L luminol and 10 μmol horseradish peroxidase (HRP). Cells (5 × 10^6) were activated by the addition of phorbol myristate acetate (PMA) (1 μg/mL) at 37°C and superoxide production measured using a Berthold (Wildbad, Germany) model 953 luminometer at 10-second intervals after a 2-minute preincubation. For the cytochrome c assay, cells (5 × 10^6) were harvested and suspended in lysis buffer with CaCl2 and MgCl2 at a final concentration of 2 × 10 μmol/L cells/mL in the presence of 100 μmol cytochrome c and 150 μmol NADPH. Superoxide production was stimulated by the addi-
tion of PMA (1 μg/mL). Reactions were incubated with gentle agitation at 37°C. For each data point a parallel reaction containing 500 ng of superoxide dismutase (SOD) was used as a control. Aliquots, 0.5 mL, were taken from each reaction at 10-minute intervals and the reaction terminated by addition of 1 mL of ice-cold PBS, and centrifugation for 4 minutes (5,000 rpm, 4°C) in a microcentrifuge (Eppendorf 5415, Hamburg, Germany). The extent of SOD inhibitable cytochrome c reduction was estimated by differential absorbance at 550 nm and 557 nm (isosbestic point).

Western blot analyses. Cells were disrupted by 2 x 5 second bursts of sonication. Crude protein extracts (30 μg) were separated on 12.5% polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Blots were incubated with antibodies to p67-phox as described previously.19 Reacting bands were visualized by reaction with 125I protein A and autoradiography. Densitometric analysis was performed using an LKB (Uppsala, Sweden) Ultroscan XL densitometer using low-density autoradiographic exposures to ensure a linear response range.

Northern blot analysis. RNA was separated on 12% agarose/formaldehyde gels, transferred to nylon membrane (Amersham Hybond-N, Buckingham, UK) using 20X SSC (1X SSC = 0.15 mol/L NaCl, 0.015 mol/L Na citrate), and hybridized overnight with a 32P-labeled p47-phox cDNA probe. Blots were washed once (10 minutes) with 2X SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature and twice (20 minutes each) in 0.1X SSC, 0.1% SDS at 60°C. Blots were air dried and autoradiographed for 3 days with intensifying screens.

Southern blot analysis. Genomic DNA samples (10 μg) were digested overnight with XbaI (2 U/μg), separated on 1% agarose-TAE gels and transferred to nylon membrane (Amersham Hybond N+) by alkaline transfer. Hybridization was at 42°C overnight in buffer containing 50% formamide, using labeled p47-phox cDNA. After hybridization blots were washed as described for the Northern blotting and autoradiographed for 16 hours with intensifying screens.

RESULTS

Superoxide generation of transduced cells. A producer cell line making recombinant pBabe Neo 47ΔA (Fig 1) retroviral particles was used to transduce EBV-immortalized B lymphocytes isolated from a p47-phox-deficient CGD patient by cocultivation. After approximately 6 weeks of growth in continuous selection (2 mg/mL G418) the transduced cells were tested for their ability to produce superoxide in response to stimulation with PMA (Fig 2). When assayed by chemiluminescence23 (Fig 2a), the untransduced cells (CGD−) failed to produce detectable superoxide, either in the presence or absence of PMA. In contrast, the retrovirally transduced cells (CGD1+, CGD2+) produce significant quantities of superoxide compared with a normal B-cell line. This superoxide generation was found to be absolutely dependent on PMA stimulation (CGD+, PMA−). A similar analysis of the same recipient cells transduced with the retroviral vector genome alone failed to detect any superoxide (data not shown). Superoxide generation was quantified using cytochrome c reduction23 (Fig 2b). The retrovirally transduced cells (CGD1+, CGD2+) produced approximately 30% (0.38 nmol/107 cells/min) and 36% (0.47 nmol/107 cells/min) of the activity of a typical normal B-cell line (1.3 nmol/107 cells/min), respectively (similar experiments on a number of our normal lines have demonstrated superoxide production in the range of 0.5 to 2.0 nmol/107 cells/min). The untransduced recipient cells (CGD2−) produced virtually no superoxide (<0.05 nmol/107 cells/min). Similar results have also been obtained with transduced cells from another p47-phox-deficient patient (not shown).

Presence and activity of retroviral vector genome in transduced cells. Having established that the transduced cells were indeed producing superoxide, the presence and activity of the p47-phox-containing retroviral vector genome was demonstrated by a combination of Southern, Northern, and Western blotting. Western blotting (Fig 3a) using a highly specific rabbit antiserum raised against the C-terminal 13 amino acids of p47-phox19 showed a protein species of 47 Kd in both the normal and transduced cells (lanes n and +) but not in the untransduced line (lane −). However, a similar antiserum directed against the p67-phox component of the oxidase detected a 67-Kd species in all three samples, confirming the integrity of the protein samples. The level of expression of p47-phox protein in these cells was estimated by densitometry (on a less intense exposure of the blot shown here, to ensure linearity of response), using the level of p67-phox to normalize for differences in protein loadings. The transduced cells were found to contain approximately 52% of the amount present in the normal B-cell line, which is itself comparable with the level of expression observed in myeloid cells.

Expression of the RNA encoding p47-phox was detected by Northern blot using a p47-phox cDNA probe (Fig 3b). In
In this instance, as expected, all three samples showed the presence of the endogenous p47-phox 1.4-kb mRNA. Only the transduced cells (lane +) contained a single additional RNA species of about 5.1 kb (the predicted size of a retroviral transcript) that hybridized to the p47-phox probe. Confirmation that the transduced cells contain an RNA species with covalently linked gag and p47-phox sequences was provided by an RNase protection assay (not shown).

Finally, the presence of the retroviral genome in the transduced cells was demonstrated by Southern blot using the cDNA probes employed in the Northern blot analysis. Genomic DNA from all the samples was digested with DNAse I. All samples showed the presence of the retroviral genomic DNA. The 19-kb band present in all lanes corresponds to hybridization of the endogenous p47-phox gene. The band at 4.9 kb corresponds to hybridization of the recombinant retroviral genomic DNA.
XbaI, which cuts once in each viral LTR and thus generates a 4.9-kb fragment regardless of the site of integration in the polyclonal transduced-cell population (see Fig 1). The copy number of transduced genomes was approximated from the strength of this signal relative to that of the endogenous p47-phox gene, which generates an XbaI fragment of about 19 kb. A 4.9-kb hybridizing band was found only in the transduced cells (Fig 3c, lane +). Taking the endogenous gene as an internal standard for two copies per cell, the relative band intensities would indicate the average copy number of transduced genomes to be close to one copy per cell. Reconstructions using the retroviral plasmid DNA (not shown) also gave a similar result.

DISCUSSION

B lymphocytes transformed by EBV represent a useful model system for the study of CGD. Though the quantity of superoxide produced by these cells is much less than that of neutrophils, it can be readily assayed by chemiluminescent methodologies and it is possible to get reasonable quantitative estimates from reduction of cytochrome c. As such, this system lends itself well to the development of approaches to the functional correction of CGD by gene transfer. It is somewhat curious that similar experiments previously reported elsewhere failed to detect production of superoxide in transduced B cells.\(^2^4\) Significantly, in those experiments, in contrast to the ones reported here, multiple RNA species containing p47-phox specific sequences were observed. This may have contributed to the substantially lower amount of p47-phox protein synthesized in their transduced cells. In this context, an encouraging factor was the effectiveness with which we were able to restore function with essentially single-copy transduced genomes. It remains to be determined whether the pBabe encoded enhancer/promoter will function as efficiently in cells of myeloid origin.

Evaluation of CGD patient survival over the last 20 years still indicates a 50% mortality rate at age 20.\(^2^5\) Whilst conventional antibiotic treatment and prophylactic use of \(\gamma\)-interferon\(^2^6\) for CGD have improved its prognosis, treatment and eradication of infection remain serious problems. In addition, success with bone marrow transplantation in this disease has been, at best, sporadic.\(^2^7\) Given this situation, CGD must be considered a good candidate disorder for treatment by somatic gene therapy.\(^2^6,2^8\) The restoration of superoxide generation to a B-cell line deriving from an autosomal recessive CGD patient reported here represents a material step forward in the evolution of gene-transfer-based therapy for chronic granulomatous disease.

ACKNOWLEDGMENT

We thank Dr Hartmut Land (ICRF) for the gift of the pBabe vector DNA, Dr Mary Collins for much helpful advice, and Peter Franklin and Berthold Instruments for their generosity in making a Model 953 Luminometer available during the course of these experiments. Thanks also to Drs Gordon Stewart, Mary Collins, and Christine Kinnon for their comments on the manuscript.

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