ORIGINAL ARTICLE

Residual NADPH Oxidase and Survival in Chronic Granulomatous Disease

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ABSTRACT

BACKGROUND

Failure to generate phagocyte-derived superoxide and related reactive oxygen intermediates (ROIs) is the major defect in chronic granulomatous disease, causing recurrent infections and granulomatous complications. Chronic granulomatous disease is caused by missense, nonsense, frameshift, splice, or deletion mutations in the genes for $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ (autosomal chronic granulomatous disease), or $gp91^{phox}$ (X-linked chronic granulomatous disease), which result in variable production of neutrophil-derived ROIs. We hypothesized that residual ROI production might be linked to survival in patients with chronic granulomatous disease.

METHODS

We assessed the risks of illness and death among 287 patients with chronic granulomatous disease from 244 kindreds. Residual ROI production was measured with the use of superoxide-dependent ferricytochrome *c* reduction and flow cytometry with dihydrorhodamine oxidation assays. Expression of NADPH oxidase component protein was detected by means of immunoblotting, and the affected genes were sequenced to identify causal mutations.

RESULTS

Survival of patients with chronic granulomatous disease was strongly associated with residual ROI production as a continuous variable, independently of the specific gene affected. Patients with mutations in $p47^{phox}$ and most missense mutations in $gp91^{phox}$ (with the exception of missense mutations in the nucleotide-binding and heme-binding domains) had more residual ROI production than patients with nonsense, frameshift, splice, or deletion mutations in $gp91^{phox}$. After adolescence, mortality curves diverged according to the extent of residual ROI production.

CONCLUSIONS

Patients with chronic granulomatous disease and modest residual production of ROI have significantly less severe illness and a greater likelihood of long-term survival than patients with little residual ROI production. The production of residual ROI is predicted by the specific NADPH oxidase mutation, regardless of the specific gene affected, and it is a predictor of survival in patients with chronic granulomatous disease. (Funded by the National Institutes of Health.)

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Chronic granulomatous disease, first described in the 1950s,[1] is a rare genetic disease (approximately 1 case in 200,000 people) without ethnic preference. The risk of death among persons with chronic granulomatous disease is 1 to 5% per year, and the extent of risk has been thought to depend on whether inheritance is an autosomal recessive trait or an X-linked trait.[2] Chronic granulomatous disease is caused by defects in any one of five subunits of phagocyte-derived NADPH oxidase, including gp91^{phox} (*CYBB* [cytochrome b-245, beta polypeptide], in approximately 65% of patients), p22^{phox} (*CYBA* [cytochrome b-245, alpha polypeptide], <5%), p47^{phox} (*NCF1* [neutrophil cytosolic factor 1], approximately 30%), p67^{phox} (*NCF2* [neutrophil cytosolic factor 2], <5%), and p40^{phox} (*NCF4* [neutrophil cytosolic factor 4], one case identified)[3]; in rare cases there is an association with glucose-6-phosphate dehydrogenase.[4] In patients with chronic granulomatous disease, the production of superoxide anion and other reactive oxygen intermediates (ROIs) by neutrophils, monocytes, macrophages, and eosinophils is impaired, leading to recurrent infections, granulomatous complications, and premature death.

Although the functioning of phagocyte NADPH oxidase is impaired in all patients with chronic granulomatous disease, there is variability among patients in the phagocytic production of residual ROIs (0.1 to 27.0% of the normal range). Analysis of this residual ROI production allows for the separation of patients with chronic granulomatous disease into subgroups with different mortality rates.

Methods

Isolation of Neutrophils and Mononuclear Cells

We obtained heparinized blood specimens from both healthy subjects and patients with chronic granulomatous disease starting in 1993. Participants provided written informed consent in accordance with clinical protocols approved by the National Institutes of Health. Peripheral-blood polymorphonuclear neutrophils and mononuclear cells were harvested from diluted blood with the use of discontinuous gradient centrifugation.[5]

Activity of NADPH Oxidase and Detection of Components

Production of neutrophil-derived ROI by patients with chronic granulomatous disease was measured with either a dihydrorhodamine oxidation assay (a myeloperoxidase-dependent[6] measure of hydrogen peroxide production), or superoxide-dependent ferricytochrome *c* reduction stimulated by phorbol myristate acetate. ROI production was stable over time (for details, see section 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).[5] For the dihydrorhodamine assay, the duration of stimulation with phorbol myristate acetate (100 ng per milliliter) was 15 minutes; the duration of the ferricytochrome *c*–superoxide quantitation was extended to 60 minutes to increase sensitivity. NADPH oxidase protein subunits in neutrophils from patients with chronic granulomatous disease were detected by means of immunoblotting (Fig. 1 in the Supplementary Appendix).[5]

Gene-Sequence Analysis

Genomic DNA was isolated with the use of the Gentra Puregene kit (Qiagen) and was sequenced by means of standard techniques. (For details, see section 2 and Tables 1 and 2 in the Supplementary Appendix.)

Statistical Analyses

For univariate and multivariate analyses based on ROI production, the amounts of superoxide and the mean fluorescence intensity of dihydrorhodamine were transformed to their common (base-10) logarithms. Factors associated with mortality were evaluated with the use of standard log-rank Kaplan–Meier methods and Cox proportional-hazards regression.[7] Time-to-event calculations were based on the birth date and the date of final follow-up or death. The values for superoxide and dihydrorhodamine were dichotomized in Cox regression analyses; hazard ratios and associated 95% confidence intervals were reported. Assumptions underlying the Cox proportional-hazards regression models were routinely evaluated with the use of Schoenfeld's residuals.[7] Standard *t*-tests, logistic regression, and clustering methods were also used.[8,9] Statistical analyses were performed with the use of SAS, version 9.1 (SAS Institute); S-Plus, version 8.0 (TIBCO); and R, version 2.9.2 (R Development Core Team), software.

Results

Characteristics of Study Participants

Demographic and survival data plus certain clinical variables previously correlated with survival (history of liver abscesses, rise in alkaline phosphatase,

and decrease in platelet count[8]) were reviewed for 287 patients with chronic granulomatous disease from 244 kindreds. A combination of genotyping and immunoblotting showed that the group was composed of 195 patients (from 169 families) with X-linked disease (gp91^{phox} deficiency, 67.9%), 71 patients (54 families) with p47^{phox} deficiency (24.7%), 13 patients (13 families) with p67^{phox} deficiency (4.5%), and 8 patients (8 families) with p22^{phox} deficiency (2.8%). One female patient had gp91^{phox} deficiency as a result of extreme X-chromosome inactivation.[10] The autosomal recessive genotypes had a fairly equal distribution with regard to the sex of patients. At the time of the last assessment or death, the patients ranged in age from 1 to 64 years; 32 had died. Three patients died within 18 months of bone marrow transplantation, and 15 others who underwent bone marrow transplantation were included among the survivors. The censoring of data for these 18 patients at the time of transplantation did not alter the overall interpretation of the survival data.

RESIDUAL ROI PRODUCTION AND SURVIVAL

Dihydrorhodamine measures the oxidation of dihydrorhodamine 123 to rhodamine 123 in phorbol myristate acetate–stimulated polymorphonuclear neutrophils. In exploratory analyses, we investigated the relationship between the dihydrorhodamine mean fluorescence intensity and 60-minute superoxide production in 197 patients for whom we had data on both dihydrorhodamine and superoxide production (Fig. 1A). Correlation between $\log_{10}$ superoxide production and $\log_{10}$ dihydrorhodamine mean fluorescence intensity ($r=0.67$, $P<0.001$) confirmed the expected covariation between the two measures. The data were empirically separated into two distinct subpopulations on the basis of superoxide production (<2.3 vs. ≥2.3 nmol per 10^6 cells per hour) and dihydrorhodamine fluorescence (<225 vs. ≥225 arbitrary units [AU]). We then applied a "partitioning around medoids" clustering algorithm[9] to assort the data into two clusters. On the basis of ROI production alone, there was 91% agreement between the two approaches. Ten of 11 patients (Fig. 1A) who died fell into the lower left quadrant, reflecting the lowest ROI values. Dichotomized ROI values separated the survival groups ($P=0.002$) (Fig. 1B). Cox regression analysis, performed with the use of the empirical discriminators listed above, showed that these variables, considered together, have a strong predictive association with survival ($P=0.008$) (section 3 and Table 3 in the Supplementary Appendix).

Using superoxide production as the sole predictor, we fit Cox regression models to survival data from 227 patients with chronic granulomatous disease in exploratory analyses. Patients were first ranked into four discrete quartiles according to neutrophil superoxide production (Fig. 1C), without consideration of their disease phenotype. Survival increased with higher superoxide production (Fig. 1C, inset, and Fig. 2A). Taking the quartile with the lowest superoxide production (quartile 1) as the reference group, we observed graded increases in the hazard ratios corresponding to increasing superoxide production in quartiles 2, 3, and 4. When $\log_{10}$ superoxide production was considered as a continuous covariate, the strength of this relationship increased ($P<0.001$; likelihood ratio chi-square=11.08, 1 degree of freedom). These findings showed that higher residual superoxide production was a predictor of longer survival.

The significant morbidity associated with chronic granulomatous disease is related to granuloma formation and obstruction of viscus organs, especially the gastrointestinal and genitourinary tracts. Fifty-five patients had gastrointestinal obstruction, but gastrointestinal complications were not associated with the level of ROI production (Fig. 2 in the Supplementary Appendix). A history of liver abscess, a declining platelet slope (a loss of more than 9000 platelets per cubic millimeter per year), and increases in the level of alkaline phosphatase (>0.25 U per liter per year) have been identified as risk factors independently associated with mortality.[8] Univariate analysis of dihydrorhodamine fluorescence in 139 patients confirmed that lower values were significantly associated with mortality (<225 AU vs. ≥225 AU) ($P=0.02$) (Table 1). In bivariate analyses that included dihydrorhodamine values, both a history of liver abscess and increases in alkaline phosphatase as covariates were significantly associated with mortality, independently of dihydrorhodamine values of less than 225 AU. In contrast, the association of a declining platelet slope with a poor outcome was not significant after adjustment for dihydrorhodamine values of less than 225 AU. Notably, patients who had dihydrorhodamine fluorescence values below this level were more likely to have a declining platelet slope (odds ratio, 2.50; 95% confidence interval, 1.09 to 5.73; $P=0.03$).

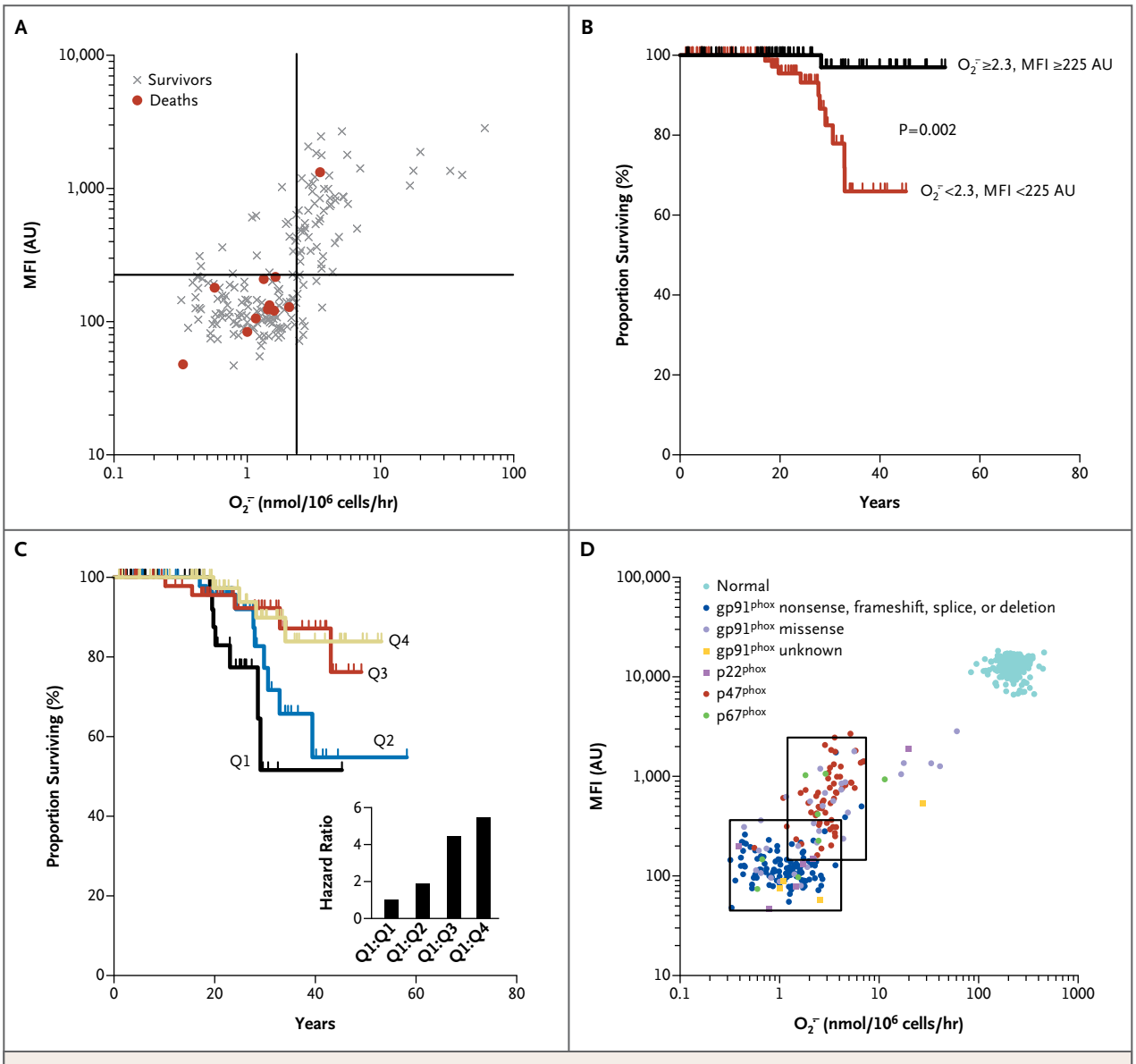


Figure 1. Production of Reactive Oxygen Intermediates and Survival in Chronic Granulomatous Disease.

A scatter plot (Panel A) shows the relationship between the production of superoxide (O_2^-) and the mean fluorescence intensity (MFI) of dihydrorhodamine in polymorphonuclear neutrophils from patients with chronic granulomatous disease. The empirical discriminator for the x axis was set at 2.3 nmol of O_2^- per 10^6 cells per hour and that for the y axis at an MFI of 225 arbitrary units (AU). Kaplan–Meier survival curves (Panel B) were calculated on the basis of the empirical separation of patients into two distinct populations according to the neutrophil production of reactive oxygen intermediates (determined on the basis of separations depicted in Panel A without consideration of the specific genotype). A Kaplan–Meier survival plot (Panel C, upper left) separates the 227 study patients into quartiles according to residual O_2^- production, without consideration of genotype. The bar graph (Panel C, lower right) shows the increases in hazard ratios when quartile 1 (Q1) is compared with the other quartiles (Q2, Q3, and Q4). The ranges of O_2^- production for Q1, Q2, Q3, and Q4 are 0.26 to 0.94, 0.95 to 1.67, 1.70 to 2.71, and 2.72 to 60.5, respectively. The data shown in Panel D represent the specific subunit mutation of each patient in Panel A; data from normal subjects were added. The black rectangles enclose 2 SD for O_2^- production and the MFI of dihydrorhodamine in patients with nonsense, frameshift, splice, or deletion mutations in $gp91^{phox}$ (lower left) and in patients with mutations in $p47^{phox}$ (upper right), respectively.

This suggests that a declining platelet slope, which is a marker of noncirrhotic portal hypertension, is related to ROI production, whereas the contributions of a history of liver abscess and alkaline phosphatase elevations to mortality were independent.

A Quartile Characteristics

Characteristic	Q1	Q2	Q3	Q4
Mean ±SE O_2^-	0.60±0.02	1.32±0.03	2.20±0.04	7.42±1.37
O_2^- range	0.26–0.94	0.95–1.67	1.70–2.71	2.72–60.5
Mean/median age of quartile (yr)	15.9/17.5	24.2/23.2	24.8/24.0	25.9/24.6
Age range of quartile (yr)	1.1–45.3	3.9–58.2	1.3–49.2	1.0–53.1
Mean/median age of survivors (yr)	14.9/16.1	23.6/22.5	24.8/24.3	25.8/22.8
No. of deaths	7	9	5	4
Mean/median age at death (yr)	22.8/20.2	27.6/28.0	25.2/24.0	26.8/26.6
Hazard ratio	1.00	1.90	4.45	5.49
95% CI	—	0.69–5.20	1.36–15.58	1.55–19.40
P value (hazard ratio vs. Q1)	—	0.21	0.014	0.008

B Quartile Subtype Composition

	Q1	Q2	Q3	Q4
	no. of patients			
All patients	56	57	57	57
Patients with subtype				
p22^{phox} (N=7)	2	2	2	1
p67^{phox} (N=7)	2	2	2	1
p47^{phox} (N=59)	1	5	21	32
P<0.001				
gp91^{phox} (N=154)	51	48 (2)	32 (1)	23 (1)
P<0.001				
Nonsense, frameshift, splice, or deletion (N=111)	44	41	21	5
P<0.001				
Missense (N=39)	7	5	10	17
P=0.007				
Missense amino acids 1–309 (except His222) (N=20)	0	0	6	14
P<0.001				
Missense amino acids 310–570 (plus His222) (N=19)	7	5	4	3

Figure 2. Defining Characteristics and Subtype Composition of the Study Quartiles for Patients with Chronic Granulomatous Disease.

Characteristics of the study quartiles are listed in Panel A. The subtype composition of each quartile is shown in Panel B. Differences in the proportion of the subtypes in the quartiles were determined with the use of a chi-square test for trend. Orange blocks with an upward slope indicate significant increases in subtype composition, and blue blocks with a downward slope significant decreases in subtype composition. Patients with gp91^{phox} chronic granulomatous disease were divided into two subgroups: one for patients with nonsense, frameshift, splice, or deletion mutations and another for patients with missense mutations. Patients with missense mutations were further divided into two additional subgroups: one for patients with missense mutations in amino acids 1 through 309 (except His222) and another for patients with missense mutations in amino acids 310 through 570 (plus His222). The numbers in parentheses in the row for gp91^{phox}, columns for Q2, Q3, and Q4, refer to patients whose mutations in gp91^{phox} could not be determined with the use of standard approaches.

GENOTYPING

Among the 273 patients who underwent complete genotyping, 154 distinct mutations were found (Fig. 3A, and Tables 4 through 7 in the Supplementary Appendix) distributed along the entire length of the gp91^{phox} gene, *CYBB*; 55 mutations were novel,[11] including 45 X-linked missense and nonsense mutations. Fewer nonsense than missense mutations were noted, but many nonsense mutations occurred in multiple kindreds. More than 50% of the nonsense, missense, or splice mutations in *CYBB* were C→T (or its complementary G→A) conversions (Fig. 4 in the Supplementary Appendix), suggesting that many mutations arose from deamination of 5-methylcytosine to thymine, as reported for other diseases.[12] For nonsense mutations, 80% were C→T (or G→A) conversions at CpG dinucleotides. Mutational hotspots were identified at *CYBB* c.676C→T (p.Arg226X), c.217C→T (p.Arg73X), c.388C→T (p.Arg130X), and c.469C→T (p.Arg157X) (Fig. 3 in the Supplementary Appendix). Two male patients with 388C→T (R130X) had somatic mosaicism, with levels of circulating normal cells of less than 10%. Five patients with *CYBB* mutations had nonsense hotspot mutations that were apparently de novo, since their mothers had only one peak in the dihydrorhodamine assay and had normal *CYBB* genotypes. Two brothers had *CYBB* promoter mutations that preserved normal NADPH oxidase activity in eosinophils but abrogated activity in neutrophils (Patients gp91-1a and gp91-1b) (Table 4 in the Supplementary Appendix), confirming a previous report.[13]

Frameshift mutations (deletions or insertions) were also randomly distributed throughout *CYBB*. Splice mutations occurred mostly at canonical intron splice-donor (GT) or acceptor sites (AG), resulting in deletions of exons from messenger RNA (Fig. 3A, and Fig. 3 in the Supplementary Appendix). However, several splice mutations that have been reported previously (in Patients gp91-17,[14] gp91-33,[15] gp91-142a,[14] and gp91-147a[14]) and one mutation that has not been reported (in Patient gp91-125a) (Table 4 in the Supplementary Appendix) created new splice sites that resulted in either insertions or deletions within the exons, out-of-frame triplet codons, or premature termination of gp91^{phox}. Eight kindreds had no detectable exons of *CYBB*, and in six patients, the large deletion extended into the adjacent *XK*, resulting in the McLeod syndrome.[16]

Table 1. Results of Univariate and Bivariate Cox Regression Models Showing the Association between Dihydrorhodamine Fluorescence Values and Mortality among Patients with Chronic Granulomatous Disease.

Model Covariates	Hazard Ratio for Death (95% CI)	P Value
Model 1		
Dihydrorhodamine <225 AU	5.46 (1.26–23.8)	0.02
Model 2		
Dihydrorhodamine <225 AU	4.83 (1.34–10.6)	0.01
History of liver abscess	3.76 (1.11–21.0)	0.04
Model 3		
Dihydrorhodamine <225 AU	4.89 (1.12–21.3)	0.04
Increase in alkaline phosphatase >0.25 U/liter/yr	5.49 (2.05–14.7)	0.001
Model 4		
Dihydrorhodamine <225 AU	4.52 (1.01–20.2)	0.05
Decline in platelet slope >9000 platelets/mm^3/yr	1.56 (0.56–4.36)	0.40

* AU denotes arbitrary unit, and CI confidence interval.

Seventy-one patients (54 kindreds) had p47^{phox} deficiency (Table 5 in the Supplementary Appendix). Complementary DNA sequencing in 61 patients revealed that 59 were homozygous and 2 were heterozygous for the common GT deletion at the start of exon 2 of *NCF1*.[17,18] The second mutation in the 2 heterozygous patients was not identified by the described sequencing approach.

Patients with p22^{phox} deficiency or p67^{phox} deficiency had missense, nonsense, splice, or deletion mutations in *CYBA* and *NCF2*, respectively, and most were homozygous (Tables 6 and 7 in the Supplementary Appendix). Previously unreported mutations in p22^{phox} were noted in three patients (p22-4, p22-5, and p22-7) (Table 6 in the Supplementary Appendix). Similarly, previously unreported mutations in p67^{phox} occurred in six patients (p67-1, p67-2, p67-4, p67-6, p67-8, and p67-9) (Table 7 in the Supplementary Appendix).

GENOTYPE, PHENOTYPE, AND RESIDUAL ROI

Neutrophil protein expression and residual ROI production were determined for each mutation (Tables 4 through 7 in the Supplementary Appendix). The gp91^{phox} protein was detected in polymorphonuclear neutrophils from less than half the patients with X-linked disease who had *CYBB* missense mutations (Fig. 3B), suggesting that most

CYBB missense mutations impair protein stability or translational efficiency. The neutrophils from all patients with p47^{phox} deficiency lacked detectable levels of p47^{phox} protein. Most patients with p22^{phox} and p67^{phox} deficiency had no detectable protein production. One patient with p67^{phox} deficiency (p67-9) (Table 7 in the Supplementary Appendix) had levels of p67^{phox} production that were close to normal.

Because gp91^{phox} has several discrete functional domains, we explored the missense mutations in more detail. Neutrophils from patients with missense mutations affecting amino acids 1 to 309 (with the exception of heme-binding His222) had a mean (±SE) level of superoxide production that was 10 times as high as that in neutrophils from patients with missense mutations affecting amino acids 310 to 570, a region that includes the binding domains of FAD (flavin adenine dinucleotide) and NADPH (14.42±4.67 nmol per 10^6 cells per hour vs. 1.53±0.24 nmol per 10^6 cells per hour, P<0.001 (Table 2 and Fig. 3B). Although previous schemes for denoting gp91^{phox} mutations have relied on protein expression, we found that missense mutations in the predicted nucleotide-binding domains (amino acids 310 to 570) often had little effect on protein expression but completely abrogated enzymatic function.[19-21] Therefore, gp91^{phox} protein expression is not a reliable indicator of residual ROI production. In general, patients with nonsense, frameshift, splice, or deletion mutations in gp91^{phox} had neither superoxide production nor protein expression (Table 2 and Fig. 3B, and Table 4 in the Supplementary Appendix).

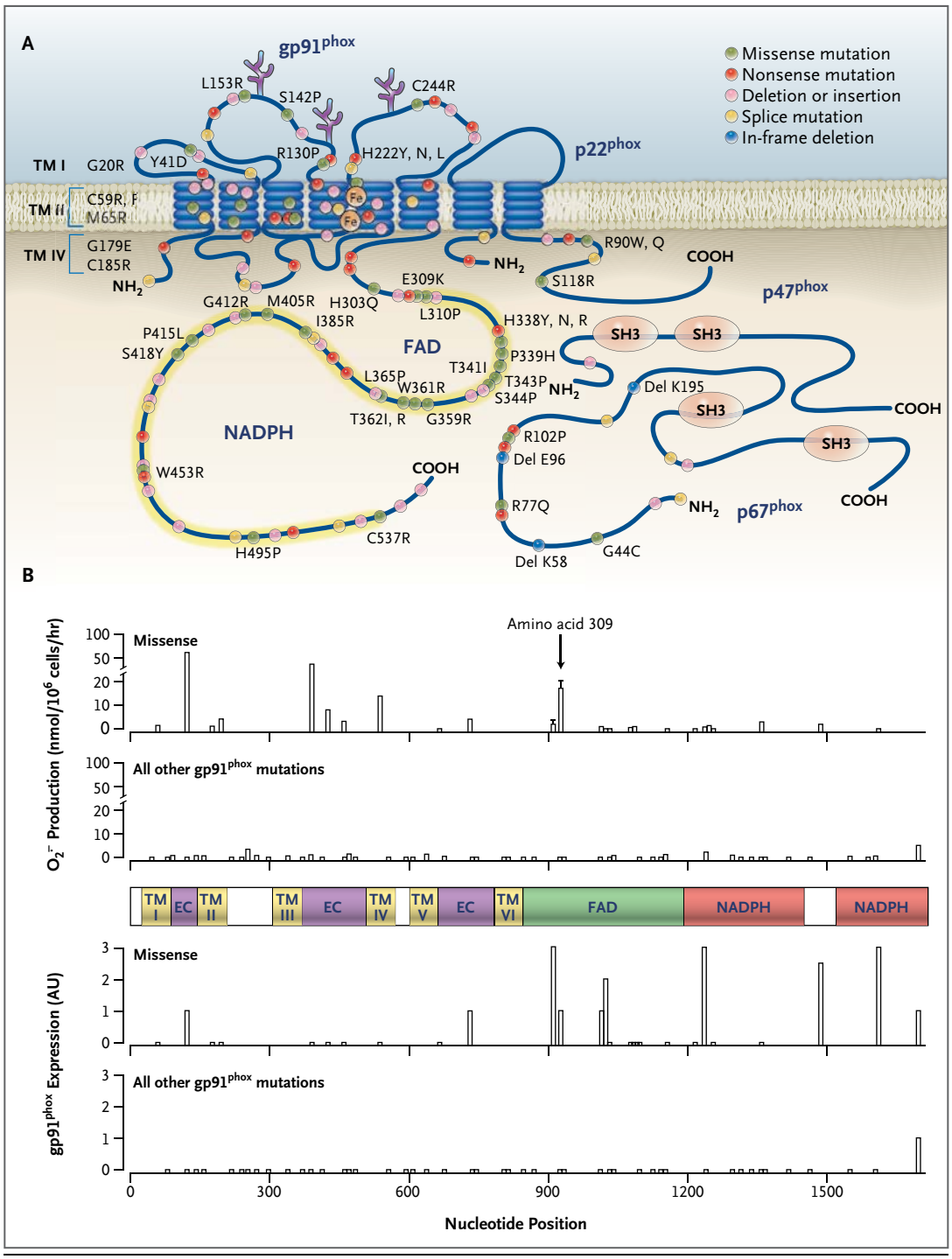
Residual ROI production in neutrophils from patients with p47^{phox} deficiency was greater than that in patients with nonsense, frameshift, splice, or deletion mutations in gp91^{phox} (P<0.001). Overlaying patient genotype on the scatter plot in Figure 1A revealed that patients with nonsense, frameshift, splice, or deletion mutations in gp91^{phox} generally had the lowest ROI production (Fig. 1D). Patients with p47^{phox} deficiency and many patients with gp91^{phox} missense mutations were located above and to the right of the lowest ROI producers in Figure 1D, reflecting higher ROI production (Table 2, and Tables 4 and 5 in the Supplementary Appendix). In the quartile analysis (Fig. 1C and 2B), 44 patients in quartile 1 had nonsense, frameshift, splice, or deletion mutations in gp91^{phox}, as compared with 5 patients in quartile 4. This was accompanied by reciprocal increases in the number of patients with p47^{phox} deficiency (1 in quartile 1 vs. 32 in quartile 4) and missense mutations in gp91^{phox} (7 in quartile 1 vs. 17 in quartile 4), especially missense mutations in gp91^{phox} affecting amino acids 1 to 309 (0 in quartile 1 vs. 14 in quartile 4). The range of ROI production in patients with p22^{phox} deficiency or p67^{phox} deficiency varied widely, with the highest production seen in patients with missense mutations; the patients in quartile 4 with these deficiencies had missense mutations (Fig. 2B).

Figure 3 (facing page). Distribution of Mutations in gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} and the Consequences of Mutations in gp91^{phox}.

Panel A shows the positions of all identified mutations in our cohort within the protein subunits of NADPH oxidase. Changes in amino acids caused by missense mutations and 3-nucleotide in-frame deletions are indicated according to the mutation. The yellow highlight identifies amino acids 310 to 570 of gp91^{phox}, a region in which missense mutations result in a loss of NADPH oxidase activity. For clarity, missense mutations in transmembrane regions (TM I, TM II, and TM IV) are indicated on the left side of the panel. Panel B shows the superoxide (O_2^-) production and expression of gp91^{phox} as a function of the nucleotide position of the mutation in patients with missense mutations in gp91^{phox} as compared with patients with all other mutations. Normal O_2^- production is 226±3 nmol per 10^6 cells per hour. The domain structure of gp91^{phox} is depicted with transmembrane domains (TM I through TM VI), extracellular domains (EC), intervening cytosolic domains, and flavin adenine dinucleotide (FAD) and NADPH binding domains. The level of expression of gp91^{phox} was scored on a scale from 0 (undetectable) to 3 (normal expression) (Fig. 1 in the Supplementary Appendix). In families with multiple patients, the mean value for the family members is presented.

GENOTYPE, TYPE OF MUTATION, AND SURVIVAL

The rate of survival was lower among patients with X-linked chronic granulomatous disease than among those with autosomal recessive disease (P=0.008) (Fig. 5A in the Supplementary Appendix), confirming previous reports.[3,22] Survival among patients with gp91^{phox} missense mutations was similar to that among patients with p47^{phox} deficiency. Both groups, however, had significantly lower rates of survival than the general U.S. population[23] (P<0.001 (Fig. 5B in the Supplementary Appendix). In contrast, patients with nonsense, frameshift, deletion, or splice mutations in gp91^{phox} had shorter survival than those with mutations in p47^{phox} (P=0.008).



DISCUSSION

Survival among patients with chronic granulomatous disease has improved since its first description in the 1950s. This change is attributed to better recognition of the disease and an understanding of its pathophysiology, development of more effective antimicrobial agents,[24,25] interferon-γ prophylaxis,[26] and bone marrow transplantation.[27] Survival in our cohort of 287 patients from 244 families studied over the past 20 years at a single center shows that childhood death from chronic granulomatous disease is now uncommon, as previously described.[2,22,28] Previously reported

Table 2. Production of Reactive Oxygen Intermediates According to Mutation Associated with Chronic Granulomatous Disease.

Mutation	No. of Families	Total No. of Patients	O_2^- Production	P Value†
			nmol/10⁶ cells/hr (range)	
gp91^{phox} — missense mutations	32	39	6.76±2.18 (0.44–60.65)	—
Amino acids 1–309, except His222	13	18	14.42±4.67 (2.09–60.65)	—
Amino acids 310–570, plus His222	19	21	1.53±0.24 (0.44–4.37)	<0.001‡
gp91^{phox} — all other mutations	94	110	1.36±0.10 (0.26–6.67)	<0.001
Nonsense	35	43	1.22±0.11 (0.36–2.70)	<0.001
Frameshift	26	31	1.47±0.26 (0.26–6.67)	0.003
Splice	24	25	1.42±0.23 (0.33–4.89)	0.004
Deletion	9	11	1.40±0.17 (0.44–2.27)	0.10
p47^{phox}	44	58	2.96±0.17 (0.56–7.05)	NS
p67^{phox}	10	10	2.56±1.02 (0.20–11.43)	NS
Missense mutations	3	3	0.20, 2.90, 11.43§	—
Other mutations	7	7	1.58±0.28 (0.60–2.47)	—
p22^{phox}	7	7	3.93±2.68 (0.39–19.93)	NS
Missense mutations	1	1	19.93§	—
Other mutations	6	6	1.26±0.27 (0.39–2.18)	—

* Plus–minus values are means ±SE. Data were transformed to their common logarithms to satisfy homogeneity of variance requirements. In families with multiple members who had chronic granulomatous disease, the values for superoxide (O_2^-) production in the affected family members were averaged. Among 339 normal subjects, the mean values for basal O_2^- production and for O_2^- production after stimulation with phorbol myristate acetate were 1.18±1.06 and 226.29±3.01, respectively. NS denotes not significant.

† Unless otherwise noted, P values are for the comparison of missense mutations in gp91^{phox} with other mutations. All P values were calculated with the use of Student's t-test.

‡ The P value is for the comparison of missense mutations in amino acids 1 through 309 (excluding heme-binding His222) with missense mutations in amino acids 310 through 570 (including heme-binding His222).

§ These individual values were presented to illustrate the variable responses observed.

differences in morbidity and mortality between X-linked and autosomal chronic granulomatous disease[2,22,29] were seen in our cohort but did not account for the substantial variation in survival rates among our patients. Our data indicate that residual ROI production is more predictive of survival than the specific NADPH-oxidase gene mutation. Even modest residual ROI production (1% of normal production) confers a significant survival benefit. This finding has important implications for the treatment of chronic granulomatous disease.

The longer survival of patients with chronic granulomatous disease has revealed the cumulative effects of frequent serious infections and of a spectrum of noninfectious inflammatory and autoimmune disorders.[2,30,31] One important consequence of severe infections — of the lungs and liver, in particular — is a predisposition to subsequent infections.[32,33] Furthermore, the exaggerated inflammatory responses of chronic granulomatous disease (e.g., dysregulated cytokine production[34,35] and decreased apoptosis[36]) probably contribute to increased scar formation, wound dehiscence, stricture development, and other problems that complicate recovery.

Our data indicate that neutrophil production of residual ROI predicts the risk of death among patients with chronic granulomatous disease and that genetic analysis can, in many cases, predict ROI production. Missense and frameshift mutations in gp91^{phox} were randomly distributed throughout the gene and were generally family-specific, whereas nonsense and spontaneous mutations were concentrated at discrete CpG dinucleotide hotspots, confirming similar findings in

other diseases. Little residual ROI production was observed in patients with nonsense, frameshift, splice, or deletion mutations in gp91^{phox}. Patients with p47^{phox} deficiency had increased production of neutrophil ROI and increased survival, as compared with patients with nonfunctional gp91^{phox} mutations. Unexpectedly, patients with missense mutations affecting gp91^{phox} amino acids 1 to 309 (with the exception of heme-binding His222) had even higher residual ROI production than was observed in patients with p47^{phox} deficiency. ROI production was not correlated with protein expression. Mutations in the FAD-binding and NADPH-binding domains of gp91^{phox} may allow normal protein expression but little residual ROI production, indicating the critical role of these domains.[19-21] Whereas protein detection provides important clues to the diagnosis of chronic granulomatous disease, quantitative ROI measurement, which correlates closely with gene sequence, is more useful in determining a patient's long-term risk. However, occasional variability in ROI production and clinical outcome in patients with the same mutation — as reported here (in Patients gp91-24a, gp91-25a, and gp91-26a or Patients gp91-98a, gp-99a, and gp-99b) and elsewhere[37] — cannot be explained solely by the mutation, suggesting that epigenetic factors or other modifying genes may play an important role.[38]

Neutrophil ROI production is a measure that can be obtained at diagnosis, correlates with genotype, and is a strong predictor of overall survival in patients with chronic granulomatous disease. Early risk assessment based on ROI production provides additional guidance for the triage of patients for more aggressive therapies such as bone marrow transplantation, which has been shown in other hematologic disorders to be more effective when performed early in life.[39,40] Our results do not suggest that the use of established standard-of-care prophylaxis and periodic clinical assessment can be ignored in the case of patients in whom neutrophils produce more residual ROI. However, our finding that even small amounts of neutrophil production of ROI confer a significant survival benefit suggests that therapies promoting even small increases in ROI production may improve survival.

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