Residual NADPH Oxidase and Survival in Chronic Granulomatous Disease

Douglas B. Kuhns, Ph.D., W. Gregory Alvord, Ph.D., Theo Heller, M.B., Ch.B., Jordan J. Feld, M.D., M.P.H., Kristen M. Pike, M.S., Beatriz E. Marciano, M.D., Gulbu Uzel, M.D., Suk See DeRavin, M.D., Ph.D., Debra A. Long Priel, M.S., Benjamin P. Soule, M.D., Kol A. Zarember, Ph.D., Harry L. Malech, M.D., Steven M. Holland, M.D., and John I. Gallin, M.D.

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Background

**Chronic Granulomatous Disease**

- Rare immune deficiency, 1:200,000 without ethnic preference
- Impaired production of superoxide anion and reactive oxygen intermediates (ROIs) by neutrophils, monocytes, macrophages, and eosinophils
- Leads to recurrent infections, granulomatous disease, and premature death
Background

Background

*Chronic Granulomatous Disease*

- Caused by defect in any one of five subunits of phagocyte-derived NADPH oxidase, can be associated with G6PD
  - $\text{g}p91^{\text{phox}}$ (65% of patients, X-linked, CYBB gene)
  - $\text{p}47^{\text{phox}}$ (30% of patients, autosomal recessive, NCF1 gene)
  - $\text{p}22^{\text{phox}}$ (<5% of patients, autosomal recessive, CYBA gene)
  - $\text{p}67^{\text{phox}}$ (<5% of patients, autosomal recessive, NCF2 gene)
  - $\text{p}40^{\text{phox}}$ (one case identified, autosomal recessive)
**Background**

**Chronic Granulomatous Disease**

- Common complications include pneumonia, suppurative adenitis, subcutaneous abscess, liver abscess, osteomyelitis, sepsis, obstructive disease secondary to granulomas, IBD
- Common microorganisms are catalase-positive (staph aureus, serratia marcescens, salmonella, burkholderia cepacia)
- Early and continuous prophylaxis with antibiotics and antifungals is the standard of care
- BMT has been attempted with mixed results
Background

Mortality in CGD

- Risk of death 1-5% per year, thought to be associated with mode of inheritance (X-linked disease associated with poor prognosis)
- Factors associated with worse prognosis include ongoing therapy for refractory infection, steroid dependent granulomatous disease, non-availability of specialist care, non-compliance with antibiotic prophylaxis
- History of liver abscess, declining platelet slope, increases in alk phos identified as risk factors independently associated with mortality
- Variability in phagocyte production of residual ROIs (0.1-27% of normal range)
- Hypothesis: residual ROI production might be linked to survival
Methods

- Heparinized blood samples obtained from CGD patients and healthy controls starting in 1993
- Harvested PMNs and mononuclear cells
- Neutrophil-derived ROI production determined with dihydorhodamine (DHR) oxidation assay or superoxide-dependent ferricytochrome c reduction stimulated by phorbol myristate acetate (PMA)
- NADPH oxidase protein subunits in neutrophils detected by immunoblotting
- Genomic DNA isolated and sequenced
- Kaplan-Meier and Cox proportional hazard analysis used for mortality data
Results – Patient Characteristics

- 287 patients with CGD in 244 kindreds
- Reviewed demographic and survival data and certain clinical variables – history of liver abscesses, rise in alk phos, decrease in platelet count
- Sex distribution was equal except for X-linked CGDs
- Age range 1-64 years
- 32 patients died, rate of survival lower in X-linked disease (p=0.008)
- 3 died within 18 months of BMT and 15 survived BMT
- Censoring the 18 patients who had undergone BMT did not affect analysis
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Number of Patients</th>
<th>Number of Families</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91&lt;sub&gt;phox&lt;/sub&gt;</td>
<td>195 (males)</td>
<td>169</td>
<td>67.9%</td>
</tr>
<tr>
<td>p47&lt;sub&gt;phox&lt;/sub&gt;</td>
<td>71</td>
<td>54</td>
<td>24.7%</td>
</tr>
<tr>
<td>p67&lt;sub&gt;phox&lt;/sub&gt;</td>
<td>13</td>
<td>13</td>
<td>4.5%</td>
</tr>
<tr>
<td>p22&lt;sub&gt;phox&lt;/sub&gt;</td>
<td>8</td>
<td>8</td>
<td>2.8%</td>
</tr>
<tr>
<td>p91&lt;sub&gt;phox&lt;/sub&gt;</td>
<td>1 (female*)</td>
<td>1</td>
<td>*extreme X inactivation</td>
</tr>
</tbody>
</table>
Results

Residual ROI production and survival

- DHR measures oxidation of dihydrorhodamine 123 to rhodamine 123 in PMA-stimulated PMNs
- Investigated relationship between DHR mean fluorescence intensity (MFI) and 60-minute superoxide production in 197 patients
Case

- 7 y/o male h/o autosomal recessive CGD diagnosed at age 3 months after he developed adenopathy and recurrent abscesses with vaccinations

Comment: This assay shows a defective oxidative burst by flow cytometric evaluation of PMA stimulated and dihydrorodamine stained cells, consistent with the diagnosis of CGD.
Production of Reactive Oxygen Intermediates and Survival in Chronic Granulomatous Disease.

- Correlation between superoxide production and DHR MFI ($r = 0.67$, $p < 0.0001$)
- Data separated based on superoxide production (empirical discriminator for x-axis set at 2.3 nmol O2, for y axis set at 225 AU of MFI)
- Sorted data into two clusters using a clustering algorithm and found 91% agreement between two approaches
- 10/11 patients who died fell into the lower left quadrant (lowest ROI values)
Production of Reactive Oxygen Intermediates and Survival in Chronic Granulomatous Disease.

- Kaplan-Meier analysis
- Dichotomized ROI values separated the survival groups $P = 0.002$
Production of Reactive Oxygen Intermediates and Survival in Chronic Granulomatous Disease.

Cox regression analysis

O2 and DHR fluorescence have strong predictive association with survival (p = 0.008)

HR for O2 < 2.3 = 10.35

HR for DHR < 225 = 10.29

two variables considered together have strong predictive association even in 197 pts with only 11 deaths

<table>
<thead>
<tr>
<th>Covariate(s) in each model</th>
<th>HR*</th>
<th>95% CI*</th>
<th>HR p-value</th>
<th>LR§ p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2&lt;2.3</td>
<td>10.35</td>
<td>1.31-81.6</td>
<td>0.027</td>
<td>0.0033</td>
</tr>
<tr>
<td>DHR&lt;225</td>
<td>10.29</td>
<td>1.31-80.92</td>
<td>0.027</td>
<td>0.0033</td>
</tr>
<tr>
<td>O2&lt;2.3</td>
<td>3.78</td>
<td>0.23-63.05</td>
<td>0.35</td>
<td>0.0078</td>
</tr>
<tr>
<td>DHR&lt;225</td>
<td>3.76</td>
<td>0.23-62.48</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

*Hazard ratio  
*Confidence Interval  
§Likelihood ratio
Production of Reactive Oxygen Intermediates and Survival in Chronic Granulomatous Disease.

- Specific subunit mutation of each patient and normal controls
- Rectangles enclose 2 SD for O2 production and MFI in patients with certain mutations
Production of Reactive Oxygen Intermediates and Survival in Chronic Granulomatous Disease.

- Patients ranked into four discrete quartiles according to neutrophil superoxide production (Q1 had lowest production)
- Survival increased with higher superoxide production
- Increased HR when Q1 compared with other quartiles
Defining Characteristics and Subtype Composition of the Study Quartiles for Patients with Chronic Granulomatous Disease.

### A Quartile Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SE ( \Delta \mathrm{O}_2^- )</td>
<td>0.60 ±0.02</td>
<td>1.32 ±0.03</td>
<td>2.20 ±0.04</td>
<td>7.42 ±1.37</td>
</tr>
<tr>
<td>( \Delta \mathrm{O}_2^- ) range</td>
<td>0.26–0.94</td>
<td>0.95–1.67</td>
<td>1.70–2.71</td>
<td>2.72–60.5</td>
</tr>
<tr>
<td>Mean/median age of quartile (yr)</td>
<td>15.9/17.5</td>
<td>24.2/23.2</td>
<td>24.8/24.0</td>
<td>25.9/24.6</td>
</tr>
<tr>
<td>Age range of quartile (yr)</td>
<td>1.1–45.3</td>
<td>3.9–58.2</td>
<td>1.3–49.2</td>
<td>1.0–53.1</td>
</tr>
<tr>
<td>Mean/median age of survivors (yr)</td>
<td>14.9/16.1</td>
<td>23.6/22.5</td>
<td>24.8/24.3</td>
<td>25.8/22.8</td>
</tr>
<tr>
<td>No. of deaths</td>
<td>7</td>
<td>9</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Mean/median age at death (yr)</td>
<td>22.8/20.2</td>
<td>27.6/26.0</td>
<td>25.2/24.0</td>
<td>26.8/26.6</td>
</tr>
<tr>
<td>Hazard ratio</td>
<td>1.00</td>
<td>1.90</td>
<td>4.43</td>
<td>5.49</td>
</tr>
<tr>
<td>95% CI</td>
<td>—</td>
<td>0.69–5.20</td>
<td>1.36–15.58</td>
<td>1.35–19.40</td>
</tr>
<tr>
<td>P value (hazard ratio vs. Q1)</td>
<td>—</td>
<td>0.21</td>
<td>0.014</td>
<td>0.008</td>
</tr>
</tbody>
</table>

### B Quartile Subtype Composition

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All patients</strong></td>
<td>36</td>
<td>57</td>
<td>57</td>
<td>57</td>
</tr>
<tr>
<td><strong>Patients with subtype</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p22(^{phox}) (N=7)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>p67(^{phox}) (N=7)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>p47(^{phox}) (N=59)</td>
<td>1</td>
<td>5</td>
<td>21</td>
<td>32</td>
</tr>
<tr>
<td>gp91(^{phox}) (N=154)</td>
<td>51</td>
<td>48 (2)</td>
<td>32 (1)</td>
<td>23 (1)</td>
</tr>
<tr>
<td><strong>Nonsense, frameshift, splice, or deletion (N=111)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Missense (N=39)</strong></td>
<td>44</td>
<td>41</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td><strong>Missense amino acids 1–309 (except His222) (N=20)</strong></td>
<td>7</td>
<td>5</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td><strong>Missense amino acids 310–570 (plus His222) (N=19)</strong></td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>

- Characteristics of the quartiles, subtype composition of each quartile
- Patients with gp91phox divided into subgroups based on mutation, mutation correlated with quartile
- Significant increases (orange) and decreases (blue) in subgroup composition by mutation
- Survival increases with higher superoxide production
Distribution of Mutations in gp91\textsuperscript{phox}, p22\textsuperscript{phox}, p47\textsuperscript{phox}, and p67\textsuperscript{phox} and the Consequences of Mutations in gp91\textsuperscript{phox}.

- 154 distinct mutations found along length of gp91phox gene (CYBB)
- Many mutations arose from deamination of 5-methylcytosine to thymine
- Superoxide production and expression of gp91phox as a function of nucleotide position of the mutation
- Level of expression of gp91phox scored on a scale from 0 (undetectable) to 3 (normal levels)
- Gp91phox protein detected in less than half the pts with X-linked disease who had CYBB missense mutations
Results of Univariate and Bivariate Cox Regression Models Showing the Association between Dihydrorhodamine Fluorescence Values and Mortality among Patients with CGD.

Model 1: Univariate analysis of DHR fluorescence in 139 pts confirmed lower values significantly associated with mortality (p=0.02)

Model 2 and 3: Bivariate analysis including DHR, h/o liver abscess or increased alk phos

Model 4: Bivariate analysis including DHR and declining platelet slope

<table>
<thead>
<tr>
<th>Model Covariates</th>
<th>Hazard Ratio for Death (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrorhodamine &lt;225 AU</td>
<td>5.46 (1.26–23.8)</td>
<td>0.02</td>
</tr>
<tr>
<td>Model 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrorhodamine &lt;225 AU</td>
<td>4.83 (1.34–10.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>History of liver abscess</td>
<td>3.76 (1.11–21.0)</td>
<td>0.04</td>
</tr>
<tr>
<td>Model 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrorhodamine &lt;225 AU</td>
<td>4.89 (1.12–21.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Increase in alkaline phosphatase &gt;0.25 U/liter/yr</td>
<td>5.49 (2.05–14.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Model 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrorhodamine &lt;225 AU</td>
<td>4.52 (1.01–20.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>Decline in platelet slope &gt;9000 platelets/mm³/yr</td>
<td>1.56 (0.56–4.36)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

* AU denotes arbitrary unit, and CI confidence interval.
Production of Reactive Oxygen Intermediates According to Mutation Associated with CGD

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. of Families</th>
<th>Total No. of Patients</th>
<th>O$_2$− Production (nmol/10$^6$ cells/hr, range)</th>
<th>P Value$\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91phox missense mutations</td>
<td>32</td>
<td>39</td>
<td>6.76±2.18 (0.44–60.65)</td>
<td>—</td>
</tr>
<tr>
<td>Amino acids 1–309, except His222</td>
<td>13</td>
<td>18</td>
<td>14.42±4.67 (2.09–60.65)</td>
<td>—</td>
</tr>
<tr>
<td>Amino acids 310–570, plus His222</td>
<td>19</td>
<td>21</td>
<td>1.53±0.24 (0.44–4.37)</td>
<td>&lt;0.001$\ddagger$</td>
</tr>
<tr>
<td>gp91phox — all other mutations</td>
<td>94</td>
<td>110</td>
<td>1.36±0.10 (0.26–6.67)</td>
<td>&lt;0.001$\ddagger$</td>
</tr>
<tr>
<td>Nonsense</td>
<td>35</td>
<td>43</td>
<td>1.22±0.11 (0.36–2.70)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Frameshift</td>
<td>26</td>
<td>31</td>
<td>1.47±0.26 (0.26–6.67)</td>
<td>0.003</td>
</tr>
<tr>
<td>Splice</td>
<td>24</td>
<td>25</td>
<td>1.42±0.23 (0.33–4.89)</td>
<td>0.004</td>
</tr>
<tr>
<td>Deletion</td>
<td>9</td>
<td>11</td>
<td>1.40±0.17 (0.44–2.27)</td>
<td>0.10</td>
</tr>
<tr>
<td>p47phox</td>
<td>44</td>
<td>58</td>
<td>2.96±0.17 (0.56–7.05)</td>
<td>NS</td>
</tr>
<tr>
<td>p67phox</td>
<td>10</td>
<td>10</td>
<td>2.56±1.02 (0.20–11.43)</td>
<td>NS</td>
</tr>
<tr>
<td>Missense mutations</td>
<td>3</td>
<td>3</td>
<td>0.20, 2.90, 11.43$\ddagger$</td>
<td>—</td>
</tr>
<tr>
<td>Other mutations</td>
<td>7</td>
<td>7</td>
<td>1.58±0.28 (0.60–2.47)</td>
<td>—</td>
</tr>
<tr>
<td>p22phox</td>
<td>7</td>
<td>7</td>
<td>3.93±2.68 (0.39–19.93)</td>
<td>NS</td>
</tr>
<tr>
<td>Missense mutations</td>
<td>1</td>
<td>1</td>
<td>19.93$\ddagger$</td>
<td>—</td>
</tr>
<tr>
<td>Other mutations</td>
<td>6</td>
<td>6</td>
<td>1.26±0.27 (0.39–2.18)</td>
<td>—</td>
</tr>
</tbody>
</table>

* 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.

$\dagger$ Unless otherwise noted, P values are for the comparison of missense mutations in gp91phox with other mutations. All P values were calculated with the use of Student’s t test.

$\ddagger$ 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).

$\ddagger$ These individual values were presented to illustrate the variable responses observed.

- ROI production by mutation, comparison of mutations to gp91phox missense mutations (p-values)
- Large range of ROIs produced, even with the same mutation
Survival Curves

- Significantly lower survival for patients with X-linked vs AR CGD (p=0.008)

- Kaplan Meier curves for patients with gp91$^{\text{phox}}$ and p47$^{\text{phox}}$ mutations vs the general population (p<0.001)
Conclusions

- Modest residual ROI production (1% of normal) confers significant survival benefit

- Residual ROI production is predicted by the specific mutation and is a predictor of survival, however residual ROI production is MORE predictive of survival than the specific mutation

- ROI production was not correlated with protein expression, there was variability in ROI production and clinical outcome in patients with the same mutation
Case

- 7 y/o male h/o autosomal recessive CGD diagnosed at age 3 months after he developed adenopathy and recurrent abscesses with vaccinations
Case

- At diagnosis, started on prophylactic bactrim and itraconazole which he continued
- Age 5 developed abdominal pain with vomiting, diarrhea, and fever
- Admitted to Bronx-Lebanon Hospital where work-up was negative for infection, developed ascites and anasarca
  - EBV IgG positive, IgM negative
  - CMV IgG positive, IgM negative
  - Hep surface antigen negative, core antigen negative
  - Had received Hep A vaccine and booster
Case

- Transferred to MSH where abdominal US showed hepatomegaly and renomegaly
- Found to have elevated LFTs
- Eventually became afebrile and was sent home with negative work-up
- Continued to be followed by liver team and LFTs eventually returned to normal range
Case

- Over the next year had several bouts of fever and diarrhea, in each case thought to be viral in etiology
- Did not gain significant weight and had poor appetite
- Continued on bactrim and switched to voriconazole (from itraconazole)
- 8/10 found to have elevated ESR, CRP, and platelets, liver enzymes normal
- Suspected low grade lung infection, CT chest performed
Case

- 8/10 baseline CT chest:
  - “Several lung cysts, multiple subcentimeter lung nodules and tree-in-bud opacities are most likely secondary to an infectious etiology, given the patient’s history of chronic granulomatous disease, neoplastic etiologies are less likely.”
Case

- 10/10 presented to the ER with fever and abdominal pain, dx with infectious vs. inflammatory colitis and given antibiotics
- Stool O+P, cultures, rotavirus, shiga toxin, microsporidia, cryptosporidia, isosporidia, and C. diff negative
- Rapid strep positive
- CXR consistent with viral PNA vs. bronchiolitis
- Another CT chest was performed
CT Chest 10/10
Case

- 10/10 CT chest showed:
  - “Again visualized are several lung cysts and multiple subcentimeter lung nodules. These have a slightly changed appearance from the prior study, some nodules are slightly larger and some are slightly smaller than the previous study. These are likely secondary to infectious etiology, neoplastic and vascular etiologies are less likely.”
Case

- Abdominal pain, vomiting, and diarrhea eventually resolved and he was released from the hospital
- Continues to have poor weight gain
- Acute phase reactants remain elevated
  - ESR 90 mm/hr
  - CRP 101.3 mg/dL
- Followed by GI, due to have colonoscopy and upper endoscopy
Case – discussion points
Case – discussion points

- What is the role of ROI measurement in determining clinical care of patient?
- If ROI is more predictive of survival than mutation, would we recommend BMT for some patients based on these results?
References


