X-linked carriers of chronic granulomatous disease: Illness, lyonization, and stability

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Background: Chronic granulomatous disease (CGD) is characterized by recurrent life-threatening bacterial and fungal infections and aberrant inflammation. Mutations in CYBB cause X-linked CGD and account for 65% to 70% of cases in Western countries.

Objective: We sought to understand the clinical manifestations associated with the X-linked CGD carrier state.

Methods: We undertook a comprehensive retrospective study of 162 affected female subjects. We examined dihydrorhodamine 123 (DHR) oxidation data for percentage of X-chromosome inactivation. We correlated lyonization (%DHR+) with clinical features. Where possible, we followed %DHR+ values over time.

Results: Clinical data were available for 93 female subjects: %DHR+ values were 46% (mean) and 47% (median; SD, 24). Using the %DHR+ value as the criterion for X inactivation, 78% of patients had levels of inactivation of 20% to 80%, suggesting random inactivation that was independent of age. In contrast, carriers with CGD-type infections had median %DHR+ values of 8% (n = 14; range, 0.06% to 48%), and those with only autoimmune or inflammatory manifestations had median %DHR+ values of 39% (n = 31; range, 7.4% to 74%). Those with both infections and autoimmunity had low %DHR+ values (n = 6; range, 3% to 14%). A %DHR+ value of less than 10% was strongly associated with infections (odds ratio, 99). Strong association persisted when %DHR+ values were less than 20% (odds ratio, 12). Autoimmunity was not associated with %DHR+ values. In 2 sets of identical twins, the %DHR+ populations tracked closely over time. Although the %DHR+ populations were very similar between sisters, those between mothers and daughters were unrelated.

Conclusions: A low %DHR+ value strongly predicts infection risk in X-linked CGD carriers, and the carrier state itself is associated with autoimmunity. (J Allergy Clin Immunol 2017;:.

Key words: Superoxide, X inactivation, dihydrorhodamine flow cytometry test, autoimmunity, lyonization

Chronic granulomatous disease (CGD) is a rare genetic disorder of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in which phagocytes have impaired production of the microbicidal reactive oxidant superoxide anion and its metabolites. In addition, patients with CGD have exuberant inflammatory responses and high rates of autoimmunity. CGD results from constitutive inactivating mutations in the genes CYBB, CYBA, NCF1, NCF2, and NCF4, which encode the structural subunits of the phagocyte NADPH oxidase. X-linked CGD is caused by mutations in CYBB, encoding gp91phox, and accounts for 65% to 70% of cases in most Western countries.

Female subjects have 2 populations of neutrophils: one set expressing the paternal X chromosome, and the other set expressing the maternal one. In female mammals most genes on one randomly chosen X chromosome are silenced epigenetically early in development to allow expression of only one X-chromosome equivalent (lyonization), thereby making female X-chromosome gene dosage largely equivalent to that of male subjects. Therefore in female X-linked CGD carriers, those neutrophils with inactivation of the CYBB mutated X chromosome will have a normal respiratory burst, whereas those neutrophils with inactivation of the normal X chromosome will have a CGD phenotype. Female carriers of CYBB mutations are typically clinically unaffected because the number of cells expressing wild-type CYBB and having normal superoxide production is adequate for protection from typical CGD infections.

X-linked CGD carriers have been reported to have a variety of autoimmune manifestations, including discoid lupus erythematosus (DLE), Raynaud phenomenon, and oral aphthous ulcers. Lupus was described in an X-linked CGD carrier in 1957, long

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Supported in part by federal funds from the National Cancer Institute, National Institutes of Health, under contract no. HHSN261200800001E. Additional support was provided by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

Disclosure of potential conflict of interest: C. Frein, D. L. Fink, D. A. Long Priel, and D. B. Kuhns receive support from the National Institutes of Health (HHSN231200800001E).

The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 29, 2016; revised April 12, 2017; accepted for publication April 18, 2017.

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0091-6749

Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology

http://dx.doi.org/10.1016/j.jaci.2017.04.035
before DHR testing was developed. More recently, Cale et al described 19 cases with a high prevalence of autoimmune manifestations. De Raven et al reviewed the literature and described signs and symptoms in female X-linked CGD carriers. Their data suggested that X-linked CGD carriers have more extensive clinical manifestations than previously thought and that antimicrobial prophylaxis might be useful.

Despite the broad and growing appreciation that the X-linked CGD carrier state might be more complex than previously appreciated, no comprehensive analysis of an affected population has been performed, making assessment of risk and guidelines for management difficult to assign. To better understand the X-linked CGD carrier state, we undertook a comprehensive retrospective review of our patient population, including clinical manifestations, such as infections, inflammatory, and autoimmune events, and DHR testing and its stability over time.

METHODS
Clinical information was retrieved from medical records of female relatives of patients with CGD or those with a family history of CGD between 1994 and 2015. All patients were seen and/or samples were examined at the National Institutes of Health after appropriate informed consent was obtained. All mutations were confirmed by means of protein testing, molecular testing, or both in the proband. Phagocyte NADPH oxidase activation was measured in individual cells by using flow cytometry with dihydrorhodamine 123 (DHR), a probe that fluoresces on neutrophil stimulation because of its oxidation to rhodamine. Neutrophils from female X-linked carriers of CGD exhibit 2 populations: an abnormal DHR(−) population expressing the mutant allele and a brightly staining DHR(+) population expressing the normal allele.

Female carriers were considered symptomatic when severe (ie, invasive) or recurrent (repeated over time) infections or autoimmune or inflammatory manifestations (AIMs) occurred. Those who had no invasive infections or AIMs were considered asymptomatic. AIMs were designated as confirmed diagnoses of DLE, systemic lupus erythematosus (SLE), thyroiditis, fibromyalgia, Crohn-like disease, or inflammatory bowel disease. Infections were documented by cultures, clinical features, or both in medical notes.

Neutrophil X-chromosome inactivation was determined by using phorbol 12-myristate 13-acetate–stimulated DHR analysis and classified according to the extent of lyonization. X-chromosome inactivation includes ratios up to 80:20. A skewed X-chromosome inactivation is any ratio outside of 80:20; a highly skewed ratio is outside of 90:10. The typical %DHR− value in the normal population is greater than 95%. DHR variation in normal populations is practically undetectable. However, variability within and between carriers has not been previously assessed, and therefore the intrinsic variability is not known. To estimate reliability, when available (n = 22), we determined the %DHR− value in the same subject within 2 days in the absence of any clinical or management change to calculate the variance of the method. We found the DHR value to be quite reproducible and reliable (r = 0.92, P ≤ .0001; data not shown).

Standard statistical parameters (means, medians, and ranges) were used to characterize the data. DHR values were examined by age both across and within subjects. The Fisher exact test was used to calculate the strength of association. All P values were 2-sided, and values of less than .05 were considered statistically significant. Statistical testing was performed with GraphPad Prism software (version 6 for Mac OS X; GraphPad Software, La Jolla, Calif).

RESULTS
A total of 162 X-linked CGD female carriers were identified for whom DHR data were available. From 117 families, 105 were mothers of a patient with CGD or diagnosed CGD carrier (3 of them also had sisters who were carriers), 35 were sisters of CGD probands or carriers, 8 were aunts or cousins, 7 were grandmothers, 4 were daughters of a patient with CGD, and 3 had no family member with a previous diagnosis of CGD. Median age at study was 36 years (range, 3 months to 80 years). DHR determination showed a mean of 46% DHR− cells and a median of 47% DHR+ cells (SD, 24). By using neutrophil %DHR− and %DHR+ values within 80:20 as a criterion for normal or random X-chromosome inactivation, 78% of subjects had normal inactivation independent of age (Fig 1). Five carrier women died over the course of the study. Median age at death was 46 years (range, 44–68 years); 2 women died of myocardial infarction at ages of 45 and 46 years.

Mutation spectrum
Genomic analysis detected mutations in 146 female carriers, including nonsense (n = 52), missense (n = 28), frameshift (n = 31), deletion (n = 15), splice (n = 17), and promoter (n = 1) mutations. Although most nonsense mutations and deletions had the lowest %DHR+ values, overall, there was no statistical difference between mutations and %DHR+ values. Those carriers with lower %DHR+ values had more clinical manifestations, mostly infections corresponding to nonsense, deletion, splicing, or frameshift mutations. However, the odds ratio (OR) was not statistically significant, despite a trend toward deletion and subjects with nonsense mutations having more infections (P = .076). Subjects with missense mutations had fewer complications than those with nonsense/deletion mutations, but the difference was not statistically significant (P = .06).

DHR and clinical manifestations
Clinical data were analyzed for those who had them recorded (n = 93). Forty-five (48%) members of the population reported symptoms based on either infections, AIMs, or both. In addition, 14 (15%) women had clinical presentations that were not specifically CGD but were clearly not asymptomatic, here denoted as “miscellaneous.” Thirty-four (37%) were assumed to be asymptomatic based on the absence of any reported problem, although this might well be an underestimate because these data were not collected prospectively (Fig 2).
Fourteen (15%) female subjects had at least 1 severe infection, and 8 of these had more than 1 severe episode or had disseminated infection. The most frequent and recurrent infections were pneumonias (10 carriers) caused by typical CGD infections, such as *Burkholderia cepacia* complex and *Aspergillus fumigatus*. Two patients had dissemination after lung infection: 1 had *Trichosporon inkin* infection, and 1 had *Nocardia* species infection. Other severe infections in this group included recurrent skin abscesses (n = 5), lymphadenitis (n = 2), osteomyelitis (n = 2), and liver abscesses (n = 2). The organisms isolated were *Serratia marcescens*, *Staphylococcus* species, *Scedosporium apiospermum*, *Actinomyces pyogenes*, and *Klebsiella pneumoniae*.

Those carriers with infections had median %DHR$^+$ values of 8% (range, 0.06% to 48%). Having a %DHR$^+$ value of less than 10% was highly associated with infection (OR, 99; P = .003). Extending the %DHR$^+$ value to less than 20% still captured significant risk, but it was less pronounced (OR, 12; P = .01). A %DHR$^+$ value of greater than 20% was not significantly associated with infection (OR, 1.24; P = .88). Two carriers had histories of infection but were asymptomatic at the time of our survey with %DHR$^+$ values of greater than 20%. However, they had not had DHR determinations during childhood at the time of their infections. One carrier was an infant with a large interstitial deletion involving ornithine transcarbamylase (OTC) and CYBB with a %DHR$^+$ value of 20% who presented with severe infections. Six of the 14 patients with severe infections also had autoimmune manifestations. Although there were a few carriers with greater than 20% %DHR$^+$ who presented with infections, the risk for greater than 20% %DHR$^+$ was not statistically significant.

**Aims**

The 31 women with AIMS had a median %DHR$^+$ value of 39% (range, 7.4% to 74%). Discoid lupus (n = 14) was the most common autoimmune manifestation, and gut involvement (n = 7) was the most common inflammatory complication. Crohn disease was diagnosed in 5 subjects with recurrent and severe manifestations, including fistulization and poor response to conventional treatment (Table I). Skin was highly affected (25%), and routine treatment was usually effective. Cutaneous and discoid lupus typically responded to first-line treatment (hydroxychloroquine) and local medication, but systemic steroids were infrequently required. For colitis, SLE, or arthritis, systemic steroids were sometimes used, and at least 2 women reported steroid complications. Three of the 5 deaths in this cohort occurred in carriers with chronic autoimmune diseases, 2 of whom (both smokers) had myocardial infarctions. Overlapping with the autoimmune disease in this cohort were photosensitivity (5 patients), oral ulcers (7 patients), nonspecific arthralgias (4 patients), alopecia (2 patients), and Raynaud phenomenon.
miscellaneous manifestations

There were 14 female subjects who were not completely asymptomatic. For the purpose of understanding any unexpected aspect of the %DHR<sup>+</sup> value, these subjects were shown in a separate group in Fig 2, but the %DHR<sup>+</sup> value of this group was overall in the normal range. Most of them had no severe viral or bacterial infections early in life. Two of them have had multiple miscarriages.

Laboratory

Routine laboratory tests were available for 107 carriers. White blood cell counts were within normal limits (median, 6700 cells/μL) outside of infections. Three carrier female subjects had lymphocytopenia (<1000 cells/μL): 1 during an acute infection (pneumonia and lymphadenitis) and 2 with granulomatous colitis; 1 had lymphocytopenia that persisted over time (total lymphocytes over time: median, 700 cells/μL; range, 130-970 cells/μL; CD19, 21-152 cells/μL; CD3, 443-1068 cells/μL; CD4, 326-707 cells/μL; CD8, 118-298 cells/μL; and natural killer, 47-114 cells/μL). Erythrocyte sedimentation rate and C-reactive protein levels were increased during infections. One of the women who died of a myocardial infarction had SLE and intermittently moderately increased sedimentation rates.

IgG (range, 805-2530 mg/dL) and IgM (range, 51-640 mg/dL) levels were overall normal to high. Two women had sporadically low IgG levels (420-600 mg/dL): one had a large interstitial deletion involving OTC, and the other had a Crohn-like disease. IgE levels were generally normal, but in 6 women mild increases were observed; 2 were more than 1500 IU/mL but without any confirmed clinical association. Antinuclear antibodies were positive in 11 of 24 carriers tested, 9 of whom had autoimmunity. Lupus anticoagulant was inconsistently positive in 3 subjects but without venous phenomena. Rheumatoid factor was positive in 5 of 36 female subjects tested.

X-chromosome inactivation and inheritance

In one pair of apparently monozygotic twins (monochorionic-diamniotic), the %DHR<sup>+</sup> value matched at 2 years of age until the last measurement at 16 years of age, changing in parallel over time (Fig 4, A). Another set of apparently identical twins was checked only at 52 years of age but also had %DHR<sup>+</sup> values of 60.4% and 62.4%, respectively (Fig 4, B).

Prompted by recognizing closely matched levels of %DHR<sup>+</sup> neutrophils in these 2 sets of apparently identical twins, we examined the patterns of X-chromosome inactivation among sisters, mothers, and grandmothers. Data were available from 27 mother-daughter pairs of 21 families. In 6 cases we had 3 generations of carriers available. There was no correlation of %DHR<sup>+</sup> cell counts found in mother-daughter pairs (correlation, 0.1; 95% CI, 0.37 to 39; Fig 4, C). Surprisingly, and in stark contrast, sisters (7 groups of siblings) were strongly correlated with each other (r = 0.78; 95% CI, 0.056 to 0.97; Fig 4, D).

**DISCUSSION**

This comprehensive retrospective clinical and lyonization analysis of CGD carriers shows a clear, strong, and probably causal relationship between skewed inactivation of the wild-type allele and the risk of infection, with that risk being dose dependent and extending robustly to at least 20% DHR<sup>+</sup> and probably beyond. In contrast, the increased susceptibility to AIM complications is unrelated to the %DHR<sup>+</sup> value but appears to be associated with the CGD carrier state per se. These data suggest that the autoimmunity and inflammation in X-linked carriers might be related to the carrier state itself and not to the overall amount of superoxide production.

Finally, we identified a surprisingly high correlation of X-chromosome inactivation between identical twins and between sisters, which was not seen at all between mothers and daughters. Two pairs of twins had strikingly similar DHR patterns. One pair changed in parallel over time, whereas the other pair had very similar %DHR<sup>+</sup> values in their 50s. These data suggest that some aspects of X-chromosome inactivation might be determined extremely early in embryogenesis. The correlation between sisters in a sibship is a novel finding. The absence of a consistent pattern of X inactivation between mothers and daughters supports a generational resetting of X inactivation. In previous studies of X-linked diseases, large deletions were more likely to cause skewing than point mutations. Although this might be true in CGD carriers as well, we analyzed only granulocytes, and therefore differences in other tissues might show different patterns.

It seems unlikely that we have underestimated the risks associated with low %DHR<sup>+</sup> values; rather, we suggest a lower limit of protection in this group. Although X inactivation can vary between tissues in healthy female subjects, the findings in this study are limited to peripheral blood neutrophil %DHR<sup>+</sup> values. More studies are needed to determine whether X inactivation in different tissues might correlate with inflammatory or autoimmune manifestations. However, it seems safe to conclude that the factors that immediately predispose to infections in both patients with CGD and X-linked carriers are linked to neutrophil superoxide production. There is clearly something about the presence of CGD neutrophils (and/or perhaps other cells without superoxide production, as well) that predisposes to AIM complications. However, that property does not depend on the actual percentage of cells impaired, suggesting that there might be immune and autoimmune reactions triggered by the presence of even a few of these cells. Whether this is related to some of the reported CGD defects in neutrophil extracellular trap

**TABLE I. AIMs in CGD carriers**

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Carriers (n)*</th>
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<tbody>
<tr>
<td>DLE</td>
<td>14</td>
</tr>
<tr>
<td>Granulomatous colitis (Crohn-like disease)</td>
<td>5</td>
</tr>
<tr>
<td>SLE</td>
<td>4</td>
</tr>
<tr>
<td>Hypothyroidism/hyperthyroidism</td>
<td>4</td>
</tr>
</tbody>
</table>

*Number of carriers with AIMs: more than 1 condition might be diagnosed in each female subject.

(3 patients). There were no %DHR<sup>+</sup> associations with symptomatic autoimmunity. The groups with either infections alone (median %DHR<sup>+</sup>, 7.2%) or autoimmunity with infections (median %DHR<sup>+</sup>, 7.75%) had significantly lower DHR values than did those who were asymptomatic or had AIMs alone (P = .001). The risk of infection is high for those with lower %DHR<sup>+</sup> values, showing a continuous increase as the %DHR<sup>+</sup> value decreases to less than 40%. However, AIM complications are unassociated with specific %DHR<sup>+</sup> values. Therefore the %DHR<sup>+</sup> value is an important predictor of infection susceptibility but not of AIMs (Fig 3).
Sex differences in susceptibility to autoimmunity have been recognized in several diseases, and skewed X-chromosome inactivation has been hypothesized to have a role in their formation, the occurrence of which has been associated with SLE, cytokine production, or inflammatory mediator degradation, is still unclear.\(^{23}\)
pathogenesis. Skin pathology accounted for almost 50% of symptomatic women in this cohort, and DLE was a common manifestation. There is a lack of solid prevalence data for DLE and cutaneous variants in the general population, but our rate of 15% is higher than in any previous population-based study. The incidence of inflammatory bowel disease in our cohort (n = 7) was also remarkable and not correlated with %DHR values. Similarly, in a large study of X-linked and recessive CGD, inflammatory bowel disease was not correlated with the degree of residual superoxide production. These data suggest that inflammatory bowel disease in patients with CGD per se is not due to lack of superoxide production but rather some autoimmune or autoimmun response associated with that defect. This population showed a broad spectrum of autoimmune or inflammation from cutaneous lupus without systemic manifestation to severe colitis.

These results are difficult to summarize into one pattern, but there is clearly an overlap with certain features of CGD. In this autoimmune and inflammatory cohort the number of patients with significantly skewed patterns of X inactivation was small, suggesting that the degree of X-chromosome inactivation was not as important as the presence of 2 populations of cells. Although it might well be relevant to monitor clinical and laboratory values over time, currently, this is probably best carried out in a research setting. Although previous studies have provided evidence for progressive skewing with age, the definition and rate of skewing has varied from study to study. In this cohort progressive skewing over time was not common but, as described in some previous cases, was clearly seen in our younger twin pair.

Battersby et al have found that X-linked carriers of CGD have more clinical disease manifestations than previously thought and more than would be predicted by their degree of lyonization if that was only predicting the CGD phenotype. Those authors hypothesized that defects in the maintained NADPH oxidase population were not the only factors in the development of symptoms and that inflammatory disorders might be driven by factors other than the absolute percentage of functioning neutrophils. Our data confirm that the association is between any degree of lyonization and inflammatory manifestations and not with low %DHR values per se. We found cutaneous manifestations at a similar prevalence to those described previously. Cale et al reported the largest series; they did not determine a correlation with specific %DHR values but reinforced the X-linked carrier state as an important risk factor for inflammatory complications.

Our data strongly support the hypothesis that infections in X-linked CGD carriers are inversely correlated with the %DHR value neutrophil counts but only consistently clinically apparent when %DHR values decrease to less than 20% and more severe as levels decrease to less than 10%. Furthermore, at least in some cases, the %DHR value can increase or decrease over time. Therefore clinical judgment will need to be exercised in deciding whom to offer prophylaxis, but it seems prudent to consider and discuss at least trimethoprim/sulfamethoxazole initiation when the %DHR value is less than 20%. In contrast, neither autoimmune nor inflammatory manifestations were correlated with %DHR values.

The broader question about the advisability of using X-linked carriers as donors for bone marrow transplantation is not directly addressed in this study. However, the demonstration of change in %DHR values over time, at least in some cases, and the association of autoimmune phenomena with the carrier state per se suggest that, where available, other donors might be preferred.

The determination of CGD carrier status is important beyond simply counseling about the risk to male offspring because it conveys important information about possible infection risk and also about risks for inflammatory and autoimmune phenomena. The %DHR value is important to determine in carrier female subjects and might be important to monitor over time.

Clinical implications: %DHR values of less than 20% in X-linked CGD carriers correlate with infection risk but not with the risk of autoimmunity and inflammation. However, %DHR values can change over time.

REFERENCES


