Cold Urticaria, Immunodeficiency, and Autoimmunity Related to PLCG2 Deletions

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This article (10.1056/NEJMoa1102140) was published on January 11, 2012, at NEJM.org.


ABSTRACT

BACKGROUND
Mendelian analysis of disorders of immune regulation can provide insight into molecular pathways associated with host defense and immune tolerance.

METHODS
We identified three families with a dominantly inherited complex of cold-induced urticaria, antibody deficiency, and susceptibility to infection and autoimmunity. Immunophenotyping methods included flow cytometry, analysis of serum immunoglobulins and autoantibodies, lymphocyte stimulation, and enzymatic assays. Genetic studies included linkage analysis, targeted Sanger sequencing, and next-generation whole-genome sequencing.

RESULTS
Cold urticaria occurred in all affected subjects. Other, variable manifestations included atopy, granulomatous rash, autoimmune thyroiditis, the presence of antinuclear antibodies, sinopulmonary infections, and common variable immunodeficiency. Levels of serum IgM and IgA and circulating natural killer cells and class-switched memory B cells were reduced. Linkage analysis showed a 7-Mb candidate interval on chromosome 16q in one family, overlapping by 3.5 Mb a disease-associated haplotype in a smaller family. This interval includes PLCG2, encoding phospholipase Cγ2 (PLCγ2), a signaling molecule expressed in B cells, natural killer cells, and mast cells. Sequencing of complementary DNA revealed heterozygous transcripts lacking exon 19 in two families and lacking exons 20 through 22 in a third family. Genomic sequencing identified three distinct in-frame deletions that cosegregated with disease. These deletions, located within a region encoding an autoinhibitory domain, result in protein products with constitutive phospholipase activity. PLCG2-expressing cells had diminished cellular signaling at 37°C but enhanced signaling at subphysiologic temperatures.

CONCLUSIONS
Genomic deletions in PLCG2 cause gain of PLCγ2 function, leading to signaling abnormalities in multiple leukocyte subsets and a phenotype encompassing both excessive and deficient immune function. ( Funded by the National Institutes of Health Intramural Research Programs and others.)
THE GENETIC DISSECTION OF UNIQUE INFLAMMATORY PHENOTYPES CAN IDENTIFY AND ELUCIDATE IMMUNOLOGIC PATHWAYS AND MECHANISMS. SUCH INVESTIGATIONS HAVE ULTIMATELY LED TO FINDINGS WHOSE SIGNIFICANCE EXTENDS BEYOND THE MONOGENIC DISEASES HARBORING THE MUTATIONS. EXAMPLES INCLUDE THE RECOGNITION THAT FOXP3 IS ESSENTIAL FOR THE DIFFERENTIATION OF REGULATORY T CELLS IN SCURFY MICE AND IN PATIENTS WITH PROFOUND IMMUNE DYSREGULATION,1,4 THE DEMONSTRATION OF A CRITICAL ROLE FOR AIRE IN THYMIC NEGATIVE SELECTION OF T CELLS IN PATIENTS WITH A SPECIFIC AUTOIMMUNE POLYENDOCRINOPIA,5 AND THE IDENTIFICATION OF NLRP3 AS A CRITICAL REGULATOR OF INTERLEUKIN-1 IN FAMILIES WITH COLD-INDUCED INFLAMMATION.6

Cold-induced urticaria is a unique inflammatory phenotype that is characterized by mast-cell degranulation and triggered by exposure to cold stimuli, a condition that can culminate in life-threatening anaphylaxis.7,8 THERE ARE VARIOUS FORMS OF COLD URTICARIA THAT DIFFER WITH RESPECT TO THE MODE OF INHERITANCE, AGES AT ONSET AND RESOLUTION, AND RESPONSES TO A RANGE OF COLD PROVOCATIVE TESTING.9 ALTHOUGH MAST CELLS HAVE BEEN CLEARLY IMPLICATED IN THE PATHOGENESIS OF COLD URTICARIA,9 THE PROCESSES LEADING TO THE DEGRANULATION OF SUCH CELLS ARE POORLY UNDERSTOOD. WE DESCRIBE THREE FAMILIES WITH COLD URTICARIA, THE GENETIC CAUSES OF THEIR DISEASE, AND THE IMPLICATIONS OF THESE FINDINGS FOR THE UNDERSTANDING OF DISEASE MECHANISMS.

METHODS

STUDY SUBJECTS

Subjects were admitted to the National Institutes of Health Clinical Center, where they were enrolled in clinical protocols approved by the institutional review board of the National Institute of Allergy and Infectious Diseases. All subjects provided written informed consent.

GENETIC ANALYSIS

We isolated genomic DNA from whole blood of members of two families and genotyped single-nucleotide polymorphisms (SNPs). We performed parametric multipoint linkage and haplotype analysis independently in each family, using Merlin software.10 We carried out whole-genome sequencing in one affected member of Family 1, as described previously.11 We screened the coding exons, splice junctions, and complementary DNA (cDNA) of PLCG2 for mutations by means of polymerase-chain-reaction (PCR) amplification of overlapping segments and then sequencing of the products using Sanger’s method. We mapped genomic deletions using long-range PCR amplification and Sanger sequencing. Additional data on assays, reagents, and primer sequences are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

EVALUATION OF PHOSPHOLIPASE Cγ2 FUNCTION

We assayed the enzymatic activity of phospholipase Cγ2 (PLCγ2) in a COS-7–cell transfection system.12 We measured intracellular calcium flux in peripheral-blood lymphocytes13 and determined degranulation of natural killer cells14 using flow cytometry. We quantified B-cell class switching in cultured peripheral-blood mononuclear cells ex vivo.15 Secondary recombination in B cells was measured by sequencing the Igκ transcript of single CD19+CD10+IgMhiCD27− transitional B cells obtained by fluorescence-activated cell sorting.16 We measured the effect of temperature on the intracellular calcium concentration in negatively selected, column-sorted, FLUO-4–stained, unstimulated B cells from the study subjects, using confocal microscopy. Laboratory of Allergic Diseases 2 (LAD2) human mast cells were transfected with mutant PLCG2 cDNA fused to green fluorescent protein, and transfected cells were then assayed for degranulation by means of flow cytometry. Additional information about assays, reagents, and statistical techniques is provided in the Supplementary Appendix.

RESULTS

DISEASE MANIFESTATIONS

We identified three independent families of European ancestry with cold urticaria and variable immune defects (Table 1, and Table S1 in the Supplementary Appendix). The cold urticaria developed at a very young age and was lifelong; the cold-related symptoms in one of these families have been described previously.7 The study subjects differed from patients with typical cold urticaria in that they had negative results on skin testing with ice-cube and cold-water immersion. However, they had positive results on skin testing for evaporative cooling (Fig. 1A, and Fig. S1 in the Supplementary Appendix) and generalized exposure to cold air, findings that are consistent with their reports.

N ENGL J MED 366;4 NEJM.ORG JANUARY 26, 2012

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that symptoms were often elicited by cold wind rather than contact with cold objects.

Of the 27 subjects who were tested, 26 had immunologic abnormalities in addition to cold urticaria (Table S1 in the Supplementary Appendix). We detected antibody deficiency in 75% of subjects who were tested, with 56% reporting a history of recurrent infection; 3 subjects had received treatment with intravenous immune globulin for severe recurrent infection and hypogammaglobulinemia. Autoantibodies or autoimmune disease was present in 56% of subjects. Granulomatous disease was observed in 7 subjects from 2 unrelated families; 3 of these subjects were born with cutaneous lesions on the fingers, nose, and ears that disappeared during infancy. The remaining 4 subjects had persistent granulomatous disease that was more than 2 SD below the age-specific mean.

**Table 1. Summary of the Clinical Manifestations of Phospholipase Cγ2–Associated Antibody Deficiency and Immune Dysregulation in the Subjects.**

<table>
<thead>
<tr>
<th>Clinical Manifestation</th>
<th>Frequency</th>
</tr>
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<tbody>
<tr>
<td>Cold urticaria</td>
<td>27/27 (100)</td>
</tr>
<tr>
<td>Recurrent sinopulmonary infection</td>
<td>12/27 (44)</td>
</tr>
<tr>
<td>Antibody deficiency*</td>
<td>15/20 (75)</td>
</tr>
<tr>
<td>Common variable immunodeficiency</td>
<td>3/27 (11)</td>
</tr>
<tr>
<td>Symptomatic autoimmune disease†</td>
<td>7/27 (26)</td>
</tr>
<tr>
<td>Positive test for antinuclear antibodies‡</td>
<td>13/21 (62)</td>
</tr>
<tr>
<td>Symptomatic allergic disease</td>
<td>15/27 (56)</td>
</tr>
</tbody>
</table>

* Antibody deficiency was defined as a serum IgM, IgG, or IgA level that was more than 2 SD below the age-specific mean.
† Symptomatic autoimmune disease was defined as the presence of any autoimmune disease, including vitiligo, autoimmune thyroiditis, inflammatory arthropitis, and undifferentiated connective-tissue disease.
‡ The test was weakly positive (1 to 3 enzyme-linked immunosorbent assay [ELISA] units) in 12 subjects and strongly positive (>3 ELISA units) in 1 subject.

**Linkage and Mutational Analysis**

Linkage analysis based on the cold urticaria phenotype in Family 1 identified a single linkage interval of 7.7 Mb on chromosome 16q21 with a logarithm of odds (LOD) score of 4.2 (Fig. 2, and Fig. S6 in the Supplementary Appendix). Whole-genome sequencing of one affected member of Family 1 did not identify any novel coding or splice-site single-nucleotide variants, insertions, or deletions within this interval. The linkage interval was narrowed by an independent analysis of Family 2 (Fig. S7 in the Supplementary Appendix), which identified a 12.6-Mb haplotype that segregated with disease status and overlapped the linkage interval in Family 1 by 3.5 Mb (Fig. 2). Among the 24 genes contained within the narrowed candidate interval, we identified PLCG2 as the primary candidate gene (Table S2 in the Supplementary Appendix). The encoded protein, PLCγ2, is a member of the phosphoinositide-specific phospholipase C family. Members of this family propagate a wide range of extracellular signals by producing diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) through hydrolysis of a phospholipid.

Of the 21 subjects who were tested for the presence of antinuclear antibodies, 13 (62%) had positive results (Table 1, and Table S1 in the Supplementary Appendix). B cells were characterized by poor in vitro expansion and reduced class-switched–antibody production after stimulation with *Staphylococcus aureus* Cowan I (SAC) and unmethylated cytosine–guanine dinucleotide (CpG)–containing oligonucleotide (Fig. 1C, and Fig. S4 in the Supplementary Appendix). Secondary recombination in CD19⁺CD10⁺IgM⁺CD27– transitional B cells was increased, as shown by the skewing of light-chain gene usage toward the terminal elements, a characteristic that is also observed in Bruton’s tyrosine kinase deficiency in patients with X-linked agammaglobulinemia, a disease that is associated with severe impairment in B-cell signaling (Fig. 1D, and Fig. S5 in the Supplementary Appendix). Unlike patients with X-linked agammaglobulinemia, who have few peripheral-blood mature B cells, subjects with PLCG2 mutations have a substantial mature B-cell compartment, which may lead to an increased occurrence of autoimmunity.
Cold Urticaria and Immunodeficiency with PLCG2 Deletions

The phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) is hydrolyzed by phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG). The IP\textsubscript{3} that is produced is responsible for calcium release from the endoplasmic reticulum, a critical event in cellular activation. There are two members of the PLC\textgamma family, PLC\textgamma\textsubscript{1} and PLC\textgamma\textsubscript{2}. Although PLC\textgamma\textsubscript{1} is expressed in many cell types, the Rac-responsive PLC\textgamma\textsubscript{2} isoform predominates and appears to have nonredundant functions in B cells\textsuperscript{19} and natural killer cells,\textsuperscript{20} both of which had abnormalities in the three families in our study.

Direct sequencing of cDNA from peripheral-blood mononuclear cells from members of Family 1 identified \textit{PLCG2} transcripts lacking exon 19 on one allele, a mutation that was present only in affected family members. Amplification of the genomic segment of \textit{PLCG2} that is flanked by exons 18 and 20 revealed a heterozygous deletion that cosegregated with cold urticaria (Fig. S8 in the Supplementary Appendix). By directly sequencing PCR products from overlapping segments flanking exon 19, we identified a deletion of 5.9 kb in \textit{PLCG2} (Fig. S8 in the Supplementary Appendix).

Using this same approach, we identified additional heterozygous deletions of \textit{PLCG2} in Families 2 and 3 (Fig. S8 in the Supplementary Appendix).
In Family 2, we identified \( \text{PLCG2} \) transcripts that lacked exons 20 through 22, caused by an 8.2-kb deletion. In Family 3, we identified \( \text{PLCG2} \) transcripts that also lacked exon 19 but were caused by a 4.8-kb deletion with breakpoints distinct from those found in Family 1. These family-specific deletions were not detected in 200 healthy subjects of Northern European ancestry. A post hoc analysis of the whole-genome sequence from 1 member of Family 1 confirmed the 5.9-kb deletion that was initially discovered on Sanger sequencing (see the Methods section in the Supplementary Appendix). We propose the term PLAI D (PLC\( \gamma_2 \)-associated antibody deficiency and immune dysregulation) to describe this unique constellation of clinical, genetic, and functional findings.

**FUNCTIONAL STUDIES OF MUTANT PLC\( \gamma_2 \)**

Each of the three deletions that were identified in these families involved the C-terminal Src-homology 2 (cSH2) domain of PLC\( \gamma_2 \) (Fig. 3A), which is...
autoinhibitory and normally prevents constitutive enzymatic function.\textsuperscript{21, 22} Transfection of COS-7 cells with PLC\textsubscript{G2} expression constructs with a deletion of the full cSH2 domain, a deletion of exon 19 (Δ19), and a deletion of exons 20 through 22 (Δ20–22) produced equivalent PLC\textsubscript{G2} activity (Fig. S9 in the Supplementary Appendix) but elevated basal and Rac-activated phospholipase activity, as compared with a nonmutant PLC\textsubscript{G2} expression construct (Fig. 3A).

Figure 3. Effects of PLAID-Associated PLC\textsubscript{G2} Deletions, Including Increased Phospholipase Activity, Deceased Cellular Activation at Physiologic Temperatures, and Enhanced Cellular Activation at Subphysiologic Temperatures.

Panel A shows enzymatic activity of phospholipase Cy\textsubscript{2} (PLC\textsubscript{Cy2}) mutants. COS-7 cells were transfected with DNA encoding either wild-type PLC\textsubscript{G2} or PLC\textsubscript{G2} with deletions of the C-terminal Src homology 2 (cSH2) domain, exon 19 (Δ19), and exons 20 through 22 (Δ20–22) (at left). PLC\textsubscript{Cy2} activity was assayed by quantification of \(^{3}H\) inositol phosphates generated by the respective enzymes (at right). Basal activity was quantified in COS-7 cells transfected with PLC\textsubscript{G2} alone, whereas the Rac-activated condition included cotransfection with a Rac2 V12 expression construct. The I bars indicate 1 SD above and below the mean release of inositol phosphate for each condition. Data are representative of three independent experiments. C2 denotes calcium-binding C2 domain, EF EF hand motif, nSH2 N-terminal SH2 domain, PH pleckstrin homology domain, SH3 SH3 domain, sPH split pleckstrin homology domain, X-box X catalytic domain, and Y-box Y catalytic domain. Panel B shows cytosplasmic calcium content (as measured by FLUO-4 staining) in natural killer (NK) cells before and after surface-receptor cross-linking (arrow indicates time of administration) in two subjects with PLAID and one control subject. Panel C shows degranulation of NK cells after incubation with sensitive target cells, as measured by cell-surface expression of CD107a, as a percent of the maximum CD107a expression observed in control NK cells. Panel D shows the effect of temperature reduction, in the absence of surface stimulation, on cytosolic calcium content in B cells sorted from a case subject and a control subject, as measured on confocal microscopy. Data points reflect the average mean fluorescence intensity of 100 individually imaged cells, and I bars indicate standard errors. Data are representative of three independent experiments. One asterisk indicates a P value of 0.01 to 0.05, two asterisks indicate a P value of 0.001 to less than 0.01, and three asterisks indicate a P value of less than 0.001. Panel E shows mast-cell degranulation, as indicated by the surface expression of lysosomal-associated membrane protein 2 (LAMP2) and measured by means of flow cytometry at 37°C (red) and 20°C (blue) in Laboratory of Allergic Diseases 2 (LAD2) human mast cells transfected with wild-type and deleted forms of PLC\textsubscript{G2}. 

\textsuperscript{21, 22}
FUNCTIONAL LYMPHOCYTE STUDIES

Despite the gain of enzymatic function caused by disruption of the autoinhibitory domain, distal signaling and PLCγ2-dependent functions were diminished in the affected family members. B cells showed defective calcium flux and phosphorylation of extracellular signal-regulated kinase (ERK) in response to surface IgM cross-linking (Fig. S10 in the Supplementary Appendix). Transfection of A20 cells with Δ19 or Δ20–22 PLCG2 resulted in reduced ERK phosphorylation stimulated by B-cell–receptor ligation, as compared with untransfected or nonmutant PLCG2-transfected cells, indicating a dominant effect of these mutations on B-cell receptor signaling (Fig. S11 in the Supplementary Appendix). In addition, natural killer cells from affected patients showed reduced calcium flux after cross-linking of activating receptors NKG2D and 2B4 (Fig. 3B) and reduced degranulation after incubation with sensitive target cells (Fig. 3C). In contrast, T-cell calcium flux triggered by CD3 cross-linking was normal (Fig. S12 in the Supplementary Appendix).

EFFECT OF COLD EXPOSURE ON MUTANT CELLS

Cytosolic calcium levels in unstimulated, purified primary B cells from subjects with PLAID steadily rose with decreasing temperature but were unchanged with cooling in nonmutant B cells (Fig. 3D). Likewise, PLAID B cells that were stimulated by B-cell–receptor cross-linking had increased ERK phosphorylation with decreasing temperatures, as compared with nonmutant B cells (Fig. S13 in the Supplementary Appendix). Mutant PLCG2-transfected A20 cells had spontaneous ERK phosphorylation at 20°C but not at 37°C (Fig. S11 in the Supplementary Appendix). Moreover, transfection with mutated PLCG2 into LAD2 mast cells, which endogenously express PLCγ2, led to spontaneous degranulation at 20°C, which was not observed in LAD2 cells transfected with nonmutated PLCG2. The degranulation was equivalent to that seen after treatment with thapsigargin (Fig. S14 in the Supplementary Appendix) but not in LAD2 cells transfected with nonmutated PLCG2 (Fig. 3E).

DISCUSSION

In three families with a dominantly inherited syndrome of cold urticaria and pleiotropic immune dysregulation, we found that genomic deletions in PLCG2 were responsible for this unique phenotype. Diminished receptor-mediated signaling in PLAID B cells resulted in abnormal activation, class-switch recombination, and receptor editing, which have been associated with antibody deficiency, recurrent infection, and impaired central tolerance. Furthermore, the signaling defect in PLAID was temperature-sensitive, as shown by the enhanced signaling and cellular activation observed at subphysiologic temperatures, even in the absence of surface-receptor ligation in B cells and mast cells expressing mutant PLCγ2.

The PLAID-associated PLCG2 deletions affected the regulatory region, including the autoinhibitory cSH2 domain, which normally couples the enzymatic activity of PLCγ2 to upstream pathways.21 PLAID-associated deletions resulted in the failure of autoinhibition and constitutive phospholipase activity, as measured by standard enzymatic assays. These data are consistent with the hypothesis that, at rest, cSH2 obstructs the PLCγ2 catalytic site and that this inhibitory interaction is relieved by phosphorylation of critical tyrosine residues, which facilitates a conformational change in PLCγ2, thereby exposing the catalytic site.21-23

Nonetheless, B cells and natural killer cells from subjects with PLAID clearly had decreased PLCγ2-dependent signaling and function, with responses similar to those observed in Plcg2-deficient mice.30 This apparently paradoxical loss of downstream function may be the direct result of chronic signaling, just as chronic B-cell–receptor stimulation results in calcium currents of diminished amplitude, alteration of signaling cascades, and ultimately, proliferative anergy.24-26 Although the specific mechanisms that underlie the reduction in PLCγ2-mediated signal transduction remain to be elucidated, there are several possible explanations. Increased phospholipase activity may lead to depletion of the substrate PIP2, proximate to PLCγ2, resulting in impaired IP3 production and IP3-mediated calcium release.27 Alternatively, excessively high concentrations of the products of PLCγ2 may induce feedback-mediated down-regulation of the distal signaling pathways.28 Both these mechanisms suggest that phospholipase inhibitors29 might be used to treat PLAID. It is also possible that the deleted regions have other functions that are critical for PLCγ2-mediated signaling, in addition to the phospholipase activity.30

Cold urticaria in patients with PLAID is characterized by mast-cell degranulation after cold stim-
Cold Urticaria and Immunodeficiency with PLG2 Deletions

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We thank Ms. Colleen Satorius for SNP genotyping and sequencing support; Ms. Julia Fekkes for her assistance in preparing the original versions of the illustrations; and the subjects and their families for their cooperation and participation in this study.

Appendix

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