Somatic Mutations in Human Ig Variable Genes Correlate with a Partially Functional CD40-Ligand in the X-Linked Hyper-IgM Syndrome

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X-linked hyper-IgM (HIGM-1) syndrome is a rare disorder resulting from mutations in the CD40-ligand (CD40L) gene. This defect is associated with normal or elevated serum levels of IgM, and with low to undetectable levels of serum IgG, IgA, and IgE. We analyzed the somatic mutation status in Ig V genes from three unrelated HIGM-1 patients by reverse-transcription PCR and sequence analysis. Two patients (B.S. and P.S.) expressed unmutated Vλ6 genes. In contrast, one patient (A.T.) was found to express mutated Vλ6 genes. Whether the presence of somatic mutations in this patient was related to a functional CD40L was assessed by deriving T cell clones from his peripheral blood cells. Upon activation, these T cell clones expressed weakly and transiently surface CD40L, and were able to induce limited isotype switch of normal naïve B cells, indicating residual CD40L function. Altogether, our results 1) confirm the central role played by CD40L in the generation of somatic mutation (patients B.S. and P.S.), 2) provide an unusual illustration of the relative dissociation between somatic mutation and isotype switching (patient A.T.), and 3) demonstrate a further complexity of the X-linked HIGM syndrome that may occur despite a partially functional CD40L.

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Table 1. Clinical features of the HIGM patients

<table>
<thead>
<tr>
<th>Age of Diagnosis</th>
<th>Age</th>
<th>Infections</th>
<th>Family History</th>
<th>Related Problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>2 years</td>
<td>Pulmonary bronchectasis, pertussis, pneumonia</td>
<td>No</td>
<td>Heart failure</td>
</tr>
<tr>
<td>PS</td>
<td>3 months</td>
<td>3 years</td>
<td>yes 1 died at 5 months of age</td>
<td>None known</td>
</tr>
<tr>
<td>AT</td>
<td>18 months</td>
<td>31 years</td>
<td>pulmonary chicken pox, measles, otitis media, gastroenteritis, pyelonephritis, fungal infections</td>
<td>3 male deaths in infancy, mother's family, male cousin died at 20 years of age</td>
</tr>
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</table>

Technologies, Bethesda, MD) in a 20-μl final volume. First-strand cDNA (3 μl) was used directly for PCR amplification.

PCR amplification

PCR reactions were conducted for 35 cycles under standard conditions (preheating 3 min at 94°C, denaturation 1 min at 94°C, annealing 2 min at 65°C, extension 3 min at 72°C) in a final volume of 100 μl containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 200 μM of each dNTP, 2.5 U Taq polymerase (GeneAmp PCR reagents kit; Perkin-Elmer Cetus, Norwalk, CT), 5% DMSO, and 100 ng of each amplification primer (Appligene, Ilkirch, France). Oligonucleotide primers used for the PCR amplification were:

5'-CD40L, GGATCCAGAAGATACCATTTCAACTT; 3'-VH6, GCACAGTAATACACAGCC; 3'-CD40L, GAAGATCTCGGATCCTGCAAGGTGACACTGTTCAG; C₅ₛ, TGGGCGCGATGACACTCC; 5'-V₅ₛ, CCCCCATCATGTCGTCCTCTTTCTAT; 3'-V₅ₛ, GCACAGTAATACACAGCC; 5'-V₅ₛ, CCCCCATCATGGGTCACAGCCATTCT.

Purification and subcloning of the PCR products

The PCR products were size fractionated by electrophoresis in 1% low melting point NuSieve GTG agarose gels (FMC Bioproducts, Rockland, ME) and recovered after gelase treatment (Epitect Technologies Corp., Madison, WI). The purified PCR products were ligated into an EcoRV-digested and dT-tailed Bluescript vector (pBluescript; Stratagene, La Jolla, CA). The ligation mixtures were transformed in MAX Efficiency Dna competency cells (Life Technologies). Bacteria were grown in Luria-Bertani medium supplemented with 50 μg/ml X-galactoside, 1 mM isopropyl-β-D-thiogalactoside; and 100 μg/ml ampicillin. Recombinant plasmids were extracted using a plasmid mini kit (Qiagen, Chatsworth, CA), digested and ddT-tailed Bluescript vector (pBluescript; Stratagene, La Jolla, CA). The ligated mixtures were transformed in MAX Efficiency Dna competency cells (Life Technologies). The ligation mixtures were transformed in MAX Efficiency Dna competency cells (Life Technologies). The ligation mixtures were transformed in MAX Efficiency Dna competency cells (Life Technologies).

Analysis of DNA sequences

The mutation in the CD40L coding gene was determined for patients B.S. and P.S., and confirmed for the previously described patient A.T. (26). IgD was expressed on >99% of the sIgD+ B cell subpopulation, as analyzed with a FACScan flow cytometer (Becton Dickinson).

Results

Clinical status of three X-linked HIGM-1 patients

The clinical features of the patients included in this study are summarized in Table I. They all suffered from recurrent episodes of pyogenic infections during early infancy, after which the diagnosis of X-linked HIGM disease was made. They all received regular i.v. injections of IgG, and antibiotics when needed. While patients A.T. and P.S. had close male relatives who died young from infections, patient B.S. developed a sporadic form of the disease. Clinical examination of patient A.T. revealed generalized lymphadenopathy, a finding commonly associated with this disorder (35). The diagnosis of X-linked HIGM was confirmed by sequencing the CD40L coding gene from each patient (Fig. 1A), including patient A.T., who was previously reported (26). Mutations affected the
extracellular CD40 binding domain of patients B.S. and P.S., causing nucleotide deletions. In B.S., this resulted in a frame shift that introduced a stop codon at aa 48. In P.S., this yielded a replacement (97 Asp to Val), followed by deletion from aa 98 to 116. As previously described, the mutation of patient A.T. results in a single amino acid substitution (36 Met to Arg) that introduces a charged residue in the CD40L transmembrane domain (26).

The VH6 and VH5 transcripts from two X-linked HIGM-1 patients are germ line. To facilitate the detection of somatic mutations, the nonpolymorphic VH6 gene was first analyzed. Transcripts were amplified with a leader VH6-specific 5' primer and with either a framework (FR) 3 VH6-specific 3' primer, or a μ-specific 3' primer. After subcloning of the amplification products, several plasmids were sequenced for each patient.

Ten VH6 clones (274 bp/clone) obtained from patients B.S. and P.S. were analyzed (Fig. 1B). Seven sequences were germ line, while three showed a single mutation. Among 21 independent VH6/VH5-Cμ rearrangements, 14 sequences were germ line and 7 displayed 1 or 2 mutations per sequence (Fig. 1C). This low frequency of mutations (3 mutations/2740 bp) is indistinguishable from the Taq error background (1 mutation/1000 bp).

The VH5 and VH6 transcripts from patient A.T. display somatic mutations

Six of six VH6 sequences derived from patient A.T. were mutated, bearing from 1 to 11 mutations (Fig. 1B). This mutation frequency (26 mutations/1644 bp analyzed, 1.6%) fell within the normal range (1.3-3.4%) established for 15 VH6 clones isolated from three control individuals (ND1, ND2, and ND3; Fig. 1B, left panel). Mutations in sequences from patient A.T. were distributed evenly between the complementarity-determining regions (CDRs) (9/450 bp, 2%) and the FR (17/944 bp, 1.8%) regions, while mutations in sequences from controls have preferentially accumulated in CDRs (54/1125 bp, 4.8%) versus FRs (43/2730 bp, 1.6%) (Table IIA). The replacement vs silent ratio of mutations (R/S) was low (4/5; R/S = 0.8) in the CDRs from

<table>
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<th>Table II. VH6 somatic mutations of patient A.T.</th>
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<tr>
<td>A. Frequency of somatic mutations^a</td>
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<tr>
<td></td>
</tr>
<tr>
<td>FRs</td>
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<td>------</td>
</tr>
<tr>
<td>AT</td>
</tr>
<tr>
<td>ND1</td>
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<tr>
<td>ND2</td>
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B. R/S ratio^b

<table>
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<tr>
<th>FRs</th>
<th>CDRs</th>
<th>VH6</th>
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</thead>
<tbody>
<tr>
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<td>0.7</td>
<td>1.2</td>
</tr>
<tr>
<td>ND1</td>
<td>1.2</td>
<td>2.5</td>
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<td>3.4</td>
</tr>
<tr>
<td>ND3</td>
<td>0.8</td>
<td>2.1</td>
</tr>
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</table>

^a Frequency of mutations was calculated by summing all of the nucleotide changes from the germ line VH6 and dividing by the total number of bases sequenced for specified portions of the VH6 gene segment.

^b R/S ratio represents the number of replacement changes divided by the number of silent changes for specified portions of the VH6 gene segment.
The clone (AT.8) (10^6 cells/ml) were stimulated with the combination of 

vant IgCl-biotinylated control mAb, followed by streptavidin PE (solid 

CD4+CD40L control T cell clone (MT9) and HIGM-1-derived T cell 

upon anti-CD3 activation (Fig. 3). Interestingly, cytoplasmic ex-

pression of CD40L was already detected 

patient A.T. when compared with the three controls (39/15, R/S = 2.6) and with the expected R/S ratio of human V_H6 CDRs (R/S = 3.9) (40).

We next analyzed 4 V_H6-μ and 6 V_5-μ transcripts from patient A.T. and 20 V_H6-μ transcripts from two normal individuals for the presence of somatic mutations (Fig. 1C). Among 10 V_5-μ/ V_H6-μ sequences derived from A.T., 5 were germ line, while the other 5 had accumulated from 1 to 7 mutations. Similarly, 7 V_H6-μ transcripts derived from controls were germ line, while the 13 others had accumulated from 1 to 13 mutations. The overall frequency of mutation in the μ transcripts was 0.8% (22/2740 bp) for patient A.T. compared with 1.2% (68/5480 bp) for the controls.

Activated T cells from patient A.T. transiently express surface CD40L

The presence of somatic mutations in the V_H gene segments from patient A.T. raised the question as to whether his CD40L molecule, despite the point mutation in its transmembrane domain, was still functional. Five T cell clones derived from the PBL of patient A.T. were found negative for CD40L when stained with the anti-TRAP antisemur, even after activation with PMA plus ionomycin, as previously described for activated PBL from this patient (26). In contrast, LL2, an anti-CD40L mAb recently generated in our laboratory, detected surface expression of CD40L by A.T.8 T cell clone after treatment with PMA and ionomycin (Fig. 2). Kinetic analysis on A.T.8 cells revealed that CD40L surface expression was detectable 3 h after stimulation and disappeared after 16 h (Fig. 3). A similar transient expression of CD40L by A.T.8 was also observed upon anti-CD3 activation (Fig. 3). Interestingly, cytoplasmic expression of CD40L was already detected 1 h after activation, and lasted for a longer period than the surface expression (Fig. 3). As expected, staining with mAb LL2 showed a CD40L surface expression by a control CD4+ T cell clone, MT9, but with different kinetics: after activation with PMA and ionomycin (Fig. 3), CD40L surface expression peaked between 3 and 10 h and was still detectable after 24 h. Of note, the signal of staining with mAb LL2 was significantly stronger than with the anti-TRAP antisemur (Fig. 2, top panel). Thus, a transient expression of CD40L was detectable on the T cell clone from patient A.T., provided that 1) early time points after activation were analyzed, and 2) an anti-CD40L mAb was used instead of the anti-TRAP antisemur.

T cell clones from patient A.T. induce low levels of CD40L-dependent proliferation and IgG production by normal naive slgD^- B cells

We tested whether the transiently expressed CD40L from patient A.T. was functional in vitro. Naive tonsilar slgD^- B cells were cocultured with anti-CD3-activated A.T.8 T cells, or with MT9 control T cells. Activated MT9 cells induced strong DNA synthesis in slgD^- cells, which was further enhanced by addition of IL-2 and IL-10 (Fig. 4). Both anti-CD40L and anti-CD40 blocking mAbs strongly inhibited this proliferation. Of note, the naive B cell proliferation induced by A.T.8 clone was always weaker (ranging from 25 to 50% of the response with MT9 T cells), and was reduced only slightly by addition of anti-CD40 or anti-CD40L mAbs, indicating that it resulted mainly from a CD40/CD40L-independent T/B interaction.

As expected, slgD^- B cells produced high levels of IgM (120 μg/ml) and of IgG (27 μg/ml) when cultured with the activated MT9 cells, in the presence of exogeneous IL-2 and IL-10 (Fig. 5, upper panel). Addition of the blocking anti-CD40L mAb (LL2) strongly and specifically inhibited IgM and IgG production, while a control mAb had no effect. slgD^- B cells cultured with anti-CD3-activated A.T.8 cells, in the presence of exogeneous cytokines, secreted low but significant levels of IgM (2.9 μg/ml) and IgG (1.2 μg/ml) (Fig. 5, lower panel). Addition of the anti-CD40L mAb LL2 almost entirely blocked the IgG production, therefore demonstrating that the CD40L from patient A.T. was at least partially functional.

Discussion

We analyzed the somatic mutation in the Ig V gene segments of three patients with X-linked HIGM-1 syndrome. Two patients possessed CD40L molecules that have almost entirely (patient B.S.) or partially (patient P.S.) lost their extracellular domain, and they expressed unmutated rearranged V_H6 transcripts. Inasmuch as somatic mutation occurs during the proliferation of centroblasts within the dark zone of germinal centers (3, 6, 41), this observation is in agreement with the reported lack of germinal centers in X-linked HIGM-1 patients (33, 34), as well as in CD40- or CD40L-deficient mice (22–24).

In contrast, somatic mutations were present in the V_H6 and V_5 transcripts of patient A.T., with a frequency comparable with that observed in normal controls. Somatic mutations were distributed evenly along the V_H6 sequences from patient A.T. However, no conclusion can be drawn regarding the Ag-driven selection process because the R/S ratios were similarly low in the CDRs and FRs of V_H6 sequences derived from A.T. and from controls (2, 4, 42–44). The lower frequency of mutations in the V_H6-μ vs V_5 transcripts from both patient A.T. and control individuals (0.8 vs 1.6% and 1.2 vs 2.4%, respectively) suggested that at least part of the V_5 transcripts was associated with secondary isotypes in both cases, but it was not possible to directly confirm this hypothesis; while γ and α transcripts were amplified after 35 cycles of PCR from the PBL of
patient A.T. using a $V_\mu$ consensus 5' primer (data not shown). neither $V_\mu$-5-Cγ nor $V_\mu$-5-Cα PCR products could be obtained.

The presence of somatic mutation exclusively in patient A.T. raised the question about a residual function of his CD40L. Indeed, A.T. is the only X-linked HIGM-1 patient reported to date to have a mutation (36 Met to Arg) in the transmembrane domain of his CD40L. This mutation was discussed previously as destabilizing the surface expression of the CD40L molecule. In keeping with this, charged residues in the transmembrane domain of molecules such as TCR or slg individual chains need to be masked to insure their proper surface expression (45, 46). As originally described for peripheral blood T cells (26), the anti-TRAP antiserum did not
allow the detection of CD40L on the surface of several CD4+ cells derived from this patient. However, LL2, a mAb directed against human CD40L, clearly detected a very transient expression of surface CD40L by cloned T cells (A.T.8) after activation. The discrepancy with the previous report can be explained by 1) the differences in the reagents used to stain CD40L, using a mAb (LL2, generated against the full molecule) or a polyclonal antiserum (anti-TRAP generated against a peptide aa 38 to 183 (26)); 2) the higher level of CD40L expression on activated T cell clones compared with freshly isolated T cells (20, 47); and 3) the down-regulation of CD40L expression on PBL in the presence of B cells (48). Our data demonstrate that the mutation in the transmembrane domain of CD40L from patient A.T. does not result in a complete suppression, but rather in a significant reduction of both the level and the duration of CD40L expression on the T cell surface after activation.

To assess the functionality of this CD40L, we tested the ability of A.T.8 T cells to induce isotype switching of slgD+ naive B cells (39). We found that activated A.T.8 T cells induced a moderate but significant IgG secretion, as well as a weak proliferation by slgD+ naive B cells. Addition of anti-CD40L mAb almost completely inhibited the IgG secretion, demonstrating a CD40L activity. In contrast, the proliferation was affected only slightly by the blocking anti-CD40L mAb, which suggests the existence of a compensatory CD40/CD40L-independent proliferation pathway in such HIGM patient.

Our findings indicate that the presence of somatic mutation correlates with the expression of a partially functional CD40L. Recently, low levels of somatic mutations in V_H genes have been reported in two of three X-linked HIGM-1 patients, but the possible CD40L activity was not assessed (49).

Interestingly, recent studies have highlighted the roles of CD40 vs CD40L signaling in B and T cell activation, respectively (25, 50, 51). CD40 triggering of the B cells plays a major role in the induction of isotype switching (51, 52), and is involved in the generation of memory B cell development (25, 51). On the other hand, it has been shown that CD40L engagement enables T cells to initiate (51) and to continue germinal center reaction (25). Therefore, how the absence (or reduction) of CD40L signaling on T cells participates in the immune defect of X-linked HIGM-1 patients remains an open and intriguing question.

An important conclusion from our study is the demonstration of a molecular heterogeneity among X-linked HIGM-1 patients that is reflected by the presence or absence of somatic mutation in their Ig V_H genes. The limited number of patients in this study did not permit us to establish a correlation between the extent of the CD40L defect and the severity of the disease. Nevertheless, our data suggest that the outcome of the CD40/CD40L interaction on B cell might depend on its duration and its intensity. While a transient interaction could be sufficient to trigger somatic mutation, a more prolonged and intense engagement of the two molecules might be required for isotype switching to occur efficiently.

Acknowledgments

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References


