

Mutations of the Ig β gene cause agammaglobulinemia in man

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Agammaglobulinemia is a rare primary immunodeficiency characterized by an early block of B cell development in the bone marrow, resulting in the absence of peripheral B cells and low/absent immunoglobulin serum levels. So far, mutations in Btk, μ heavy chain, surrogate light chain, Ig α , and B cell linker have been found in 85–90% of patients with agammaglobulinemia. We report on the first patient with agammaglobulinemia caused by a homozygous nonsense mutation in Ig β , which is a transmembrane protein that associates with Ig α as part of the preBCR complex. Transfection experiments using *Drosophila melanogaster* S2 Schneider cells showed that the mutant Ig β is no longer able to associate with Ig α , and that assembly of the BCR complex on the cell surface is abrogated. The essential role of Ig β for human B cell development was further demonstrated by immunofluorescence analysis of the patient's bone marrow, which showed a complete block of B cell development at the pro-B to preB transition. These results indicate that mutations in Ig β can cause agammaglobulinemia in man.

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Abbreviations used: BCR, B cell receptor; BLNK, B cell linker; EGFP, enhanced GFP; LC, light chain; μ HC, μ heavy chain; PE, phycoerythrin; SLC, surrogate LC.

The development of B lymphocytes from pluripotent progenitors is a tightly regulated process that occurs in hemopoietic tissues, primarily embryonic liver and bone marrow in mammals (1). In these sites, lymphoid progenitors lacking Ig expression (pro-B cells) give rise to large B lymphocyte precursors (preB cells) expressing μ heavy chains (μ HCs) (2–5) as a result of μ HC V(D)J gene rearrangements. A key checkpoint in B cell lineage development is the ability of the newly generated μ HC to associate with the surrogate light chain (SLC) composed of VpreB and λ 5/14.1 and homologous to the V and C regions of LCs (6, 7). SLC binds nascent μ HC proteins, thereby releasing them from BiP-mediated retention in the endoplasmic reticulum (8–9). SLC/ μ HC homodimers then associate with Ig α /Ig β heterodimeric signal-transducing elements to form the preB cell receptor (BCR), which is exported from the Golgi apparatus to the preB cell surface in the context of lipid rafts, where they associate with

signaling elements such as Syk, Lyn, Btk, and B cell linker (BLNK). Signaling through the pre-BCR leads to a transient cellular proliferation and the V-J_L rearrangement of the κ or λ LCs (10–12). Successful VJ_L rearrangement allows the assembly of BCRs composed of μ HC, LC, and Ig α /Ig β on newly generated immature IgM-expressing B cells (13–14), which then exit the bone marrow and complete their maturation in the secondary lymphoid organs.

The crucial role of preBCR signaling during early B lineage differentiation is indicated by the block in pro-B to preB cell differentiation in mice and man with deficiencies in single preBCR components or in the essential downstream signaling elements (15–20).

Mutations of *BTK*, which is the gene responsible for X-linked agammaglobulinemia, account for ~85% of cases with agammaglobulinemia and absent circulating B cells (21). The remaining 15% of patients constitute a heterogeneous group, including patients with mutations in μ HC, λ 5, Ig α , and BLNK, which are the genes responsible for the autosomal recessive

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forms of agammaglobulinemia. However, mutations in these genes do not account for all patients with selective defect of B cell development, suggesting that mutations in other, yet to be identified genes may affect early B cell development. Regardless of the genetic defect, patients with a selective arrest of B cell development are characterized by early onset of severe bacterial infections as a result of their inability to mount an antibody response.

To better clarify the nature of genetic defects that results in abnormal B cell development in man, we screened patients with early-onset hypogammaglobulinemia and absent circulating B cells for mutations in genes that are expressed in the early stages of B cell differentiation.

We have identified one patient with a homozygous defect in the Ig β -encoding gene, and have compared the peripheral B cell phenotype of this patient with that of patients with defects in the μ HC or Btk. Transfection experiments using *D. melanogaster* S2 Schneider cells demonstrated that the mutation in Ig β abrogates the assembly of the BCR on the cell surface. Bone marrow studies showed that the mutation causes a complete block in B cell development at the pro-B to preB transition, a phenotype resembling that observed in Ig β -null mice (22).

RESULTS AND DISCUSSION

Mutation of the Ig β gene causes agammaglobulinemia

As part of an ongoing effort to extensively genotype agammaglobulinemic patients for known and candidate genes causing agammaglobulinemia (23), we found for the first time a homozygous mutation in the Ig β -encoding gene in a patient clinically diagnosed with agammaglobulinemia. The patient, a 20-yr-old Italian male, is the first-born child of

healthy parents without known consanguinity. He has two healthy sisters, and no positive family history for primary immunodeficiencies is present in the pedigree. The pregnancy and delivery were uneventful. At the age of 8 mo, the patient was hospitalized because of pneumonia of the left lobe and *Salmonella*-caused enteritis. Immunological work up showed a marked hypogammaglobulinemia (IgG, 100 mg/d; IgA and IgM, undetectable) and absence of peripheral CD19-positive cells (<1%), in the presence of normal numbers of T and NK cells; T cell function, assessed by mitogen-induced proliferation, was normal. Intravenous immunoglobulin replacement therapy was initiated at the age of 8 mo. During follow up, despite appropriate immunoglobulin substitution therapy, the patient suffered several episodes of bacterial conjunctivitis, acute otitis media, sinusitis, and bronchitis. A computed tomography scan of the nasal sinuses showed a pattern compatible with chronic sinusitis, whereas no bronchiectasis was observed. *BTK* gene sequence analysis was normal.

Genomic DNA from this patient was analyzed by PCR amplification and direct sequencing of exons and exon-intron junctions of genes encoding for the preBCR components. We found a homozygous C>T nucleotide substitution in exon 3 of the *B29* gene, encoding for the Ig β protein (Fig. 1), at position c.238 (GenBank accession no. M89957) corresponding to amino acid 80. The mutation causes the replacement of Gln80 with a stop codon within the extracellular immunoglobulin domain of Ig β , preventing the expression of the functional transmembrane protein and possibly interfering with the assembly of the preBCR on cell surface.

Both parents of the patient were heterozygous for this mutation. The Gln80X mutation was not detected in DNA samples obtained from 90 healthy controls; in addition,

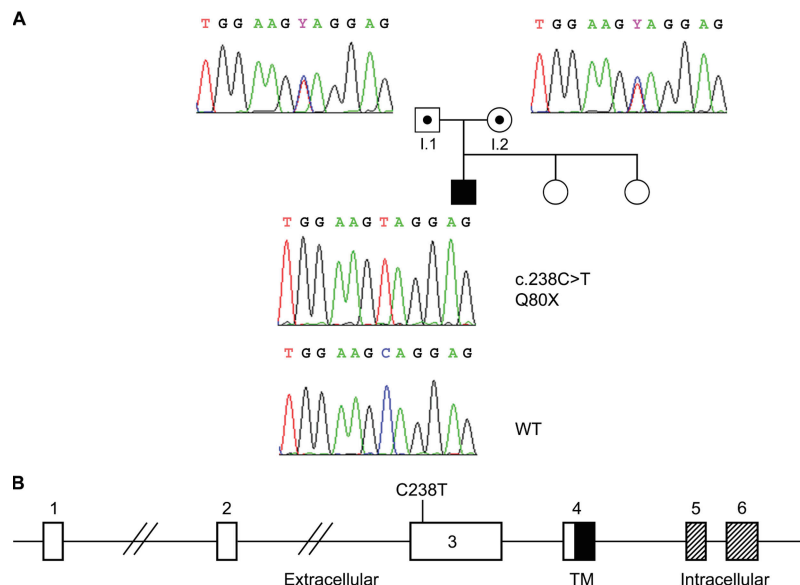


Figure 1. Mutation analysis of the *B29* gene. (A) Electropherograms from a portion of exon 3 showing the 238 C>T mutation at the base corresponding to amino acid 80 of the Ig β chain, compared with the wild-type sequence from a healthy donor (WT). The sequences obtained from heterozygous parents (I.1 and I.2) are shown. (B) Schematic representation of Ig β -encoding gene.

sequence analysis of the whole *B29* gene reveals the presence of a common haplotype represented by a T>C silent change at codon 122 in cis with a 3'-untranslated region T>C transition, which is present at a high frequency ($f_{CC} = 0.76$) in the 90 controls. Our patient is homozygous for the less common 122/T and 3'-untranslated region /T haplotype, suggesting the possibility of a founder effect for the mutated allele. Although not reported, consanguinity of patient's parents cannot be excluded.

Reconstitution of the BCR in S2 cells

It has been shown that efficient transport of IgM to the cell surface requires coexpression of Ig β ; furthermore, IgM and Ig β alone are sufficient to reconstitute antigen-specific signal transduction by Ig β (24). The effect of the Gln80X Ig β mutation on BCR assembly was assessed by a reconstitution experiment in *Drosophila melanogaster* S2 cells.

The human Ig β cDNA was cloned in an appropriate expression vector, and *D. melanogaster* S2 cells were cotransfected with mouse λ 1LC, μ HC, FLAG-tagged Ig α , and either human wild-type Ig β or mutant Gln80X Ig β . After induction of expression of the recombinant proteins, cells were harvested and reconstitution of the IgM BCR was detected by staining with a phycoerythrin (PE)-conjugated anti-FLAG antibody that binds to Ig α and a biotinylated anti-IgM antibody. PE- and Cy5-conjugated streptavidin double-positive cells indicate the expression of the BCR on the cell surface.

Fig. 2 shows that the wild-type human Ig β chain is as efficient as the mouse Ig β chain at promoting assembly of the IgM BCR complex on the cell membrane (compare the bottom left and top right graphs). On the contrary, insertion of the Gln80X mutation in Ig β prevents reconstitution of the IgM BCR on the cell surface, which is similar to expression of Ig α only (compare the bottom right and top left graphs).

S2 cells cotransfected with FLAG-tagged Ig α and wild-type Ig β and stained with the PE-conjugated anti-FLAG antibody showed that the human Ig β alone was not able to carry Ig α to the cell surface, proving that, in the aforementioned experiments, any Ig α chain detected at the S2 cell surface is, indeed, part of the assembled BCR complex (unpublished data).

Immunofluorescence studies

PBMCs from the patient carrying the Ig β mutation, three patients with Btk deficiency, three patients with μ HC deficiency, and three healthy controls were compared using standard flow cytometry markers. The dramatic reduction of circulating B cells in the Ig β -deficient patient was confirmed using the typical B cell marker CD19 (unpublished data). As expected, PBMCs from the patient, as well as PBMCs from patients with mutations in Btk and μ HC, did not express mature IgM on the cell surface (unpublished data).

The analysis of the alternative pan-B cell marker CD22 in the Ig β -deficient patient, as well as in Btk- and μ HC-deficient patients, revealed the presence of a cell population expressing

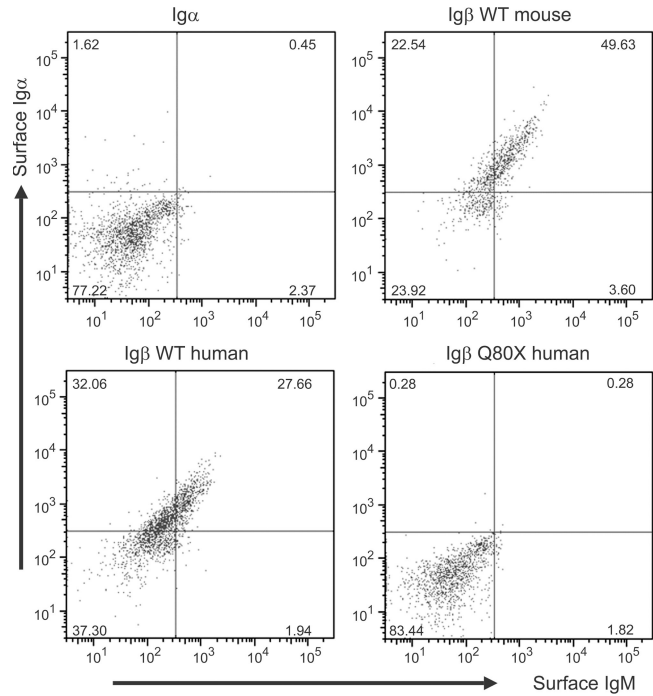


Figure 2. Surface expression of the IgM BCR in S2 cells. S2 cells were cotransfected with plasmids encoding for EGFP, mouse μ HC, λ 1 LC, Ig α , and different Ig β chains, as indicated. After 5 h, expression cells were harvested and stained with PE-labeled anti-FLAG antibody and biotinylated anti-IgM antibody, followed by streptavidin-Cy5, to detect Ig α and membrane IgM, respectively. Cells were gated for EGFP-positive cells to normalize the transfection efficiency.

CD22 at lower levels compared with healthy controls (unpublished data). The CD22^{lo} subset was poorly represented (<1%) in 25 healthy controls, whereas in patients CD22^{lo} cells range from 2 to 4.5% of the gated lymphocytes.

The CD22^{lo} cells did not express either the pan-B cell marker CD19 or the early B cell marker CD10; because the CD22 molecule was previously found expressed in human basophiles (25), we tested if the CD19⁻/CD22^{lo} population in agammaglobulinemic patients was accounted for by basophiles. This turned out to be the case, as demonstrated by Toluidine blue staining of the sorted CD22^{lo} cells, showing pure basophiles with typical large, dark granules. Furthermore, the CD22^{lo} cells were positive for CD13, which is a myelomonocytic lineage marker (unpublished data). Therefore, the CD22^{lo} cell population from the Ig β -deficient patient did not belong to the B lineage.

To determine at which stage B cell development is blocked, bone marrow cells from the patient and an age-matched control were evaluated by cytofluorimetric analysis. CD19⁺ B cells were markedly reduced in the patient (\sim 1%) when compared with the healthy control (>10%) (Fig. 3). Two-color immunofluorescence was used to define the percentages of pro-B cells (CD34⁺CD19⁻) and preB/immature B cells (CD34⁻CD19⁺). The coexpression of CD19 and CD34 showed that >85% of the patient's B cells belong to the pro-B

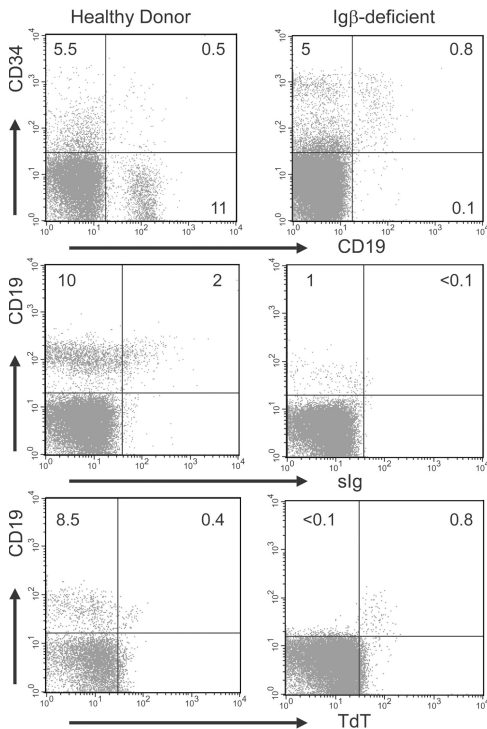


Figure 3. Cytofluorimetric analysis of B cell development. Bone marrow mononuclear cells from the Ig β -deficient patient and an age-matched healthy control were stained for surface CD19, CD34, Ig, and intracellular TdT. The patient showed a marked reduction in the number of CD19+ cells within the lymphoid gate, and >85% of the B-lineage cells were positive for CD34 (top row). Virtually none of the CD19+ cells from the Ig β -deficient patient were B cells, as defined by the coexpression of CD19 and surface immunoglobulin (middle row). Permeabilized cells were stained for cytoplasmic TdT. The majority of the CD19+ cells from the patient were pro-B cell precursors, as defined by coexpression of CD19 and TdT (bottom row).

stage of differentiation, whereas <5% of the healthy control's B cells presented the pro-B phenotype. These findings indicate a block of B cell development at the transition from the pro-B to preB stage.

To further characterize the point of arrest of B cell development, expression of TdT and surface Ig were evaluated. Remarkably, the Ig β -deficient patient presented an accumulation of TdT-positive cells that did express CD19. Furthermore, no surface immunoglobulin expression could be detected on the patient's B cells. These results further confirm that the arrest of B cell development in the Ig β -deficient patient occur at the pro-B to preB transition; a similar block at the pro-B stage has been described in patients affected by mutations of other members of the preBCR complex, i.e., μ HC deficiency (23) and Ig α deficiency (19). Unfortunately, we were unable to perform further biochemical and functional analyses, such as assessment of the extent of V(D)J recombination, because of the low number of bone marrow lymphocyte precursors obtained from the patient biopsy.

In principle, the occurrence of a stop codon in the N-terminal portion of Ig β could result in a truncated protein

lacking the transmembrane domain because of premature termination; however, it is also possible that the occurrence of a premature stop codon causes nonsense-mediated RNA decay. In any event, the mutation in Ig β prevents the assembly of the IgM BCR on the cell surface, as demonstrated by the reconstitution experiment shown in Fig. 2.

The preBCR checkpoint is essential for B cell development. So far, animal models deficient for different preBCR components were shown to have an arrest at the pro-B to preB cell stage of development, although some models presented a leaky phenotype (e.g., the presence of peripheral B cells in λ 5-deficient mice [reference 26]). Human mutations in preBCR components further elucidated the role of the preBCR complex in B cell development, and underlined that animal models are not always concordant with the phenotype of patients carrying mutations in the same gene; the aforementioned human λ 5 deficiency presents a severe defect in B cells, with absence of peripheral B cells. On the other hand, human Ig β deficiency closely resembles the phenotype observed in knockout mice in that these mice have no mature peripheral B cells and B cell development is blocked at the pro-B cell/preB cell stage (22).

In conclusion, this is the first report of a patient with agammaglobulinemia caused by a homozygous Gln80X mutation in Ig β , resulting in a stop codon that abrogates the expression of the preBCR complex on the cell surface. Bone marrow B cell analysis of the Ig β -deficient patient indicated an arrest at the pro-B–preB transition, a phenotype resembling the one observed in other known forms of agammaglobulinemia.

MATERIALS AND METHODS

Genetic analysis. Genomic DNA was extracted from peripheral blood leukocytes using standard techniques. Exons and exon–intron junctions were amplified by PCR using FastStart Taq DNA Polymerase (Roche) under standard conditions. Purified PCR products were sequenced on both strands using a Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and were run on an ABI 3730 Genetic Analyzer (Applied Biosystems). The primers used in this study are available upon request. The M89957 GenBank entry was used as reference sequence.

For the genetic diagnosis, the informed consent was signed by the patient.

DNA constructs. For inducible expression of human Ig β in S2 cells, wild-type and mutant cDNAs were cloned into the S2 plasmid pRmHa-3 containing a copper-inducible metallothionein promoter. Total RNA extracted from PBMC was amplified by RT-PCR using the following oligonucleotides: Ig β _cDNA-F 5'-GGTCGGGGACAGAGCAG-3' and Ig β _cDNA-R 5'-CGACCTGGCTCTCACTCCT-3', and the corresponding PCR fragment was subcloned into the pRmHa-3 plasmid. The C>T single-nucleotide substitution was introduced into the Ig β cDNA by site-directed mutagenesis.

Reconstitution of IgM-BCR in S2 cells. *D. melanogaster* S2 Schneider cells were grown in Schneider's *Drosophila* medium (Life Technologies, Inc.) supplemented with 5–10% FCS at 27°C with atmospheric CO₂ levels. Transient transfection of S2 cells and induction of expression (5 h) was performed as previously described in Wossning et al. (27). For FACS analysis, cells were stained with anti-FLAG PE antibody and biotinylated anti-IgM antibody, followed by Cy5-conjugated streptavidin. To normalize the transfection efficiencies, S2 cells were cotransfected with the vector pDEGFP containing the enhanced GFP (EGFP) coding sequence.

Cytofluorimetric analysis. PBMCs from fresh blood samples and bone marrow MCs from patients and healthy controls were isolated by Ficoll-Hypaque gradient centrifugation and stained with the following conjugated anti-human monoclonal antibodies: FITC-labeled anti-human CD19, PE-labeled anti-human CD22, PE-labeled anti-human CD34, PE-labeled anti-human surface Ig, and PE-labeled anti-human TdT. Cells were resuspended in PBS and 0.1% BSA at the concentration of 5×10^5 – 1×10^6 cells/ml, and then stained with and the anti-human antibodies using standard protocols. Stained cells were washed twice with PBS/BSA and analyzed with a FACS-Calibur flow cytometer (Becton Dickinson).

The human experiments have been approved by the hospital Ethical Committee of Spedali Civili of Brescia.

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The authors declare there are no conflicts of interest.

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