Noninfectious complications in patients with pediatric-onset common variable immunodeficiency correlated with defects in somatic hypermutation but not in class-switch recombination

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Background: Common variable immunodeficiency (CVID) is a heterogeneous syndrome characterized by impaired immunoglobulin production and usually presents with a normal quantity of peripheral B cells. Most attempts aiming to classify these patients have mainly been focused on T- or B-cell phenotypes and their ability to produce protective antibodies, but it is still a major challenge to find a suitable classification that includes the clinical and immunologic heterogeneity of these patients.
Objective: In this study we evaluated the late stages of B-cell differentiation in a heterogeneous population of patients with pediatric-onset CVID to clinically correlate and assess their ability to perform somatic hypermutation (SHM), class-switch recombination (CSR), or both.
Methods: We performed a previously reported assay, the restriction enzyme hotspot mutation assay (IgκREHMA), to evaluate *in vivo* SHM status. We amplified switch regions from genomic DNA to investigate the quality of the double-strand break repairs in the class-switch recombination process *in vivo*. We also tested the ability to generate immunoglobulin germline and circle transcripts and to upregulate the activation-induced cytidine deaminase gene through *in vitro* T-dependent and T-independent stimuli.
Results: Our results showed that patients could be classified into 2 groups according to their degree of SHM alteration. This stratification showed a significant association between patients of group A, severe alteration, and the presence of noninfectious complications. Additionally, 60% of patients presented with increased microhomology use at switched regions. *In vitro* activation revealed that patients with CVID behaved heterogeneously in terms of responsiveness to T-dependent stimuli.
Conclusions: The correlation between noninfectious complications and SHM could be an important tool for physicians to further characterize patients with CVID. This categorization would help to improve elucidation of the complex mechanisms involved in B-cell differentiation pathways. (J Allergy Clin Immunol 2017;139:913-22.)

Key words: *Common variable immunodeficiency, somatic hypermutation, class-switch recombination, hypermutation, pediatric, switch*

The hypogammaglobulinemia of at least 2 immunoglobulin isotypes characterizes the heterogeneous group of the primary immunodeficiency disorder known as common variable immunodeficiency (CVID; OMIM #240500).[1-5] These patients are particularly susceptible to recurrent infections of the respiratory tract, as well as the gastrointestinal system, and manifest noninfectious complications, such as autoimmunity, gastrointestinal disorders, and lymphoproliferation.[1,5]

Despite the rarity of this condition, CVID is the most common immunodeficiency of clinical significance whose main immunologic defect, the failure of immunoglobulin production, is usually associated with a normal peripheral B-cell counts.[1,5] Genetic defects associated with survival, differentiation, and/or immune interactions of B cells have been proposed to be responsible for the pathophysiology of the disease. Several attempts aiming to classify patients with CVID have mainly focused on T- or B-cell phenotypes and their ability to produce protective antibodies,[2,6,7] but it is still a major challenge to find a suitable classification that includes the clinical and immunologic heterogeneity of these patients and their variable response to treatment. The plasma IgG level has poor predictive value in patients with CVID because patients with slightly decreased IgG levels can be highly susceptible to infections and benefit from replacement with intravenous gammaglobulin therapy.[8] Nevertheless, the molecular mechanisms underlying the maturation of the antibody response triggered by natural infections and vaccines have been poorly investigated in these patients.

During somatic hypermutation (SHM), point mutations are introduced into the V region of immunoglobulin genes (both IgH as IgL), increasing antibody affinity for the antigen, and this process is essential for the generation of long-lived plasma and memory B cells.[9-11] Failure in the affinity maturation process of

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Abbreviations used
AICDA: Activation-induced cytidine deaminase
Bcl-6: B-cell lymphoma 6
CSR: Class-switch recombination
CT: Circle transcript
CVID: Common variable immunodeficiency
DSB: Double-strand break
GLT: Germline transcript
IgκREHMA: Restriction enzyme hotspot mutation assay
NHEJ: Nonhomologous end joining
SHM: Somatic hypermutation
TD: T-dependent
TI: T-independent

all isotypes of antibodies could underlie a qualitative humoral immunodeficiency. In previous reports impaired SHM was associated with a high frequency of severe respiratory tract infections in a cohort of mainly adult patients with CVID.[12] In addition, antibody response maturation also requires the class-switch recombination (CSR) process, in which the Cμ region is replaced by a downstream immunoglobulin CH gene, resulting in a change from IgM to IgG, IgA, or IgE, a mechanism involving S regions located upstream of each CH except Cδ.[13]

Both SHM and CSR are dependent on activation-induced cytidine deaminase (AICDA) function, an enzyme expressed in antigen-activated B cells that generates high-affinity antibodies and antibody CSR, respectively.[14] To achieve CSR, AICDA generates double-strand breaks (DSBs) mainly repaired through a nonhomologous end-joining (NHEJ) mechanism[15] by using little or no sequence homology. The NHEJ machinery requires a large number of factors (eg, Ku70/Ku80, DNA-PKcs, DNA ligase IV, Artemis and Cernunnos),[16-20] and defects in this mechanism lead to alternative pathways (eg, use of microhomology) in an attempt to repair DSBs at the S junctions, as shown in patients with primary immunodeficiencies involving defects in NHEJ genes.[21,22]

In this study we evaluated the capacity to perform SHM and CSR *ex vivo* and *in vitro* in a population of patients with pediatric-onset CVID whose immunologic and clinical data were previously reported,[23] allowing us to propose 3 different subsets of patients with CVID.

METHODS

Patients and control subjects

This study included 25 unrelated patients with CVID (12 male patients) with a mean age of onset of symptoms of 5.6 years (range, 1-14 years) and a mean age of diagnosis of 11.3 years (range, 4-16.1 years). Mutations in the *CD40L*, *CD40*, *AICDA*, *UNG*, *PMS2*, *DCLRE1C* (ARTEMIS), and *DNA LIG4* genes were excluded in patients presenting with normal or with increased IgM levels. Patients with decreased peripheral B-cell counts were also excluded from the study. We considered patients as belonging to the smB^+ (switched memory B cells >2%) or smB− (switched memory B cells <2%) subgroups, depending on their B-cell immunophenotype, according to the EUROclass CVID classification.[6] These patients were previously reported (Patients Part B),[23] and the main clinical and immunologic data were summarized in Tables I and II. Informed written consent was obtained from the patient or parental guardian before participation in accordance with the Declaration of Helsinki.

Twenty-two healthy donors matched for age, sex (11 male donors), and ethnic background were included in the study and provided written consent under a separate ethics protocol for healthy donors. Five cord blood samples were included as negative controls in the restriction enzyme hotspot mutation assay (IgκREHMA) and were donated after informed consent was obtained from the delivering women. The research protocol was approved by the internal ethics review board of the Hospital Garrahan.

Total RNA preparation and cDNA synthesis

Total RNA was extracted from PBMCs by using TRIzol reagent (Invitrogen, Carlsbad, Calif), and cDNA was prepared with a first-strand cDNA synthesis kit (Amersham Biosciences, Little Chalfont, United Kingdom), according to the manufacturer's instructions.

RNA from memory and naive B-cell subsets was obtained by using the RNeasy Micro Kit (Qiagen, Hilden, Germany), whereas cDNA was prepared with SuperScript III (Invitrogen), according to the manufacturer's instructions.

Human cell isolation

PBMCs were isolated by means of density centrifugation over a Ficoll-Hypaque Plus (Amersham) gradient. B cells from 7 patients and 4 healthy donors were purified from PBMCs by means of negative selection with the MACS B Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Memory and naive B-cell subsets were purified from total B cells obtained by using the FACSAria Cell Sorter (BD Biosciences, San Jose, Calif) with CD27–fluorescein isothiocyanate, IgD–phycoerythrin, and CD19–phycoerythrin-Cy7 (all from eBioscience, San Diego, Calif). All fractions were obtained with a purity of greater than 95%.

Cell cultures and reagents

Culture of PBMCs or naive B cells from patients with CVID or healthy donors was performed in complete RPMI medium supplemented with 10% (vol/vol) FBS (Gibco, Grand Island, NY). In the T-dependent (TD) stimulation cells were incubated with 500 ng/mL CD40 ligand (CD40L; PeproTech, Rocky Hills, NJ) and 200 U/mL IL-4 (Schering-Plough, Kenilworth, NJ). CD40 was cross-linked with 1 μg/mL mouse 89 mAb (Schering-Plough). Mouse IgG_1 mAb with irrelevant binding activity (Santa Cruz Biotechnology, Dallas, Tex) was used as a control. In the T-independent (TI) stimulation cells were incubated with 5 μg/mL phosphorothioate-modified 5′-tcgtcgttttgtcgttttgtcgtt-3′ oligodeoxynucleotide-2006 (Operon Technologies, Olive Branch, Miss) and 50 ng/mL IL-10 (PeproTech).

RT-PCR and quantitative RT-PCR

Quantification of human Iγ1-Cγ1 or Iα-Cα germline transcripts (GLT), switched Iγ1-Iγ2–Cμ or Iα-Cμ circle transcripts (CTs), AICDA transcript, and glyceraldehyde-3-phosphate dehydrogenase was performed from total RNA extraction in 2- or 4-day cultures with TRIzol (Invitrogen). cDNA was generated by mean of reverse transcription with Superscript II RT (Invitrogen). PCR primers and conditions used for standard or quantitative RT-PCR analysis were performed, as previously reported.[24-26] Results of qRT-PCR were normalized to *ACTB* mRNA and presented as relative expression compared with that of B cells incubated with control antibody.

Characterization of SHM

The IgκREHMA was performed, as previously described.[12]

Characterization of switch recombination junctions

Genomic DNA was purified from peripheral blood cells from patients and healthy donors by using standard methods. Amplification of Sμ-Sα from *in vivo* switched cells was performed by using a previously described nested PCR assay.[21] The PCR-amplified switch fragments were gel purified (GE Healthcare, Fairfield, Conn) and cloned into pGEM-T vector (Invitrogen), and sequence analysis was performed with DNA Sequencing Analysis software (PE Applied Biosystems, Foster City, Calif) on an ABI 3130 (Applied Biosystems). CSR junctions were determined by aligning the