Ⅲ　研究成果の刊行物・別冊

Brief report

Oligoclonal expansion of T lymphocytes with multiple second-site mutations leads to Omenn syndrome in a patient with *RAG1*-deficient severe combined immunodeficiency

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Omenn syndrome (OS) is a rare primary immunodeficiency characterized by the presence of activated/oligoclonal T cells, eosinophilia, and the absence of circulating B cells. OS patients carry leaky mutations of recombination activating genes (*RAG1* or *RAG2*) resulting in partial V(D)J recombination activity, whereas null mutations cause severe combined immunodeficiency with absence of mature T and B cells (T^-B^- SCID). Here we describe somatic mosaicism due to multiple second-site mutations in a patient with RAG1 deficiency. We found that he is homozygous for a single base deletion in the *RAG1* gene, which results in frameshift and likely abrogates the protein function. However, the patient showed typical OS features. Molecular analysis revealed that several second-site mutations, all of which restored the *RAG1* reading frame and resulted in missense mutations, were demonstrated in his T cells. These findings suggest that his revertant T-cell mosaicism is responsible for OS phenotype switched from T^-B^- SCID. (Blood. 2005; 106:2099-2101)

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Introduction

Omenn syndrome (OS) is an autosomal recessive primary immunodeficiency[1,2] and caused by mutations of the recombination activating genes (*RAG1* and *RAG2*).[3] OS mutations maintain a residual recombination activity that allows limited T-cell receptor (TCR) gene rearrangements in the thymus, whereas null mutations cause a complete block of T- and B-cell development and lead to severe combined immunodeficiency (SCID) with absence of mature T and B lymphocytes (T^-B^- SCID).[4] However, the occurrence of the same mutations in patients with T^-B^- SCID and OS suggests that "leaky" mutations in *RAG* genes may not be solely responsible for the development of OS.[5]

Somatic revertant mosaicism is a rare phenomenon that is increasingly being described in human genetic disorders.[6,7] In all cases reported to date, revertant cells carried a single revertant sequence.[6,7] It is also recognized that revertant mosaicism is an additional basis for milder phenotype in several primary immunodeficiencies such as adenosine deaminase deficiency,[8] X-linked SCID,[9] and Wiskott-Aldrich syndrome.[10] Here we describe an unusual case of RAG1 deficiency presenting somatic T-cell mosaicism due to multiple second-site mutations and show that the patient's revertant T-cell mosaicism might have contributed to the modification of his clinical features.

Study design

Patient

The patient was the second child born to consanguineous, healthy Japanese parents. He developed generalized exudative erythroderma at age 1 month, followed by failure to thrive and persistent cough. At age 2 months, the patient was hospitalized for upper respiratory infections and otitis media. Two weeks later, he suffered from sepsis due to *Pseudomonas aeruginosa*. Laboratory evaluation at age 3 months showed moderate anemia, leukocytosis (104×10^9/L [104 000/μL]) with marked eosinophilia (21.8×10^9/L [21 800/μL]), and hypogammaglobulinemia (immunoglobulin G [IgG], 1.48 g/L [148 mg/dL]; IgA, less than 0.01 g/L [less than 1 mg/dL], IgM 0.02 g/L [2 mg/dL], and IgE less than 2 kIU/L). The level of soluble interleukin-2 receptor was markedly elevated at 19 400 kIU/L (normal, 220-530 kIU/L). Immunophenotypic analysis showed the absence of peripheral B cells and marked increase of both $CD4^+$ and $CD8^+$ T cells with activated/memory phenotypes. A skin biopsy revealed lymphocytic infiltration in the upper dermis with occasional eosinophils and destruction of epidermal-dermal junction. Based on these findings, a clinical diagnosis of OS was made.

Cell isolation, sequencing, and TCRVβ repertoire

$CD4^+$ and $CD8^+$ T cells were purified using magnetic beads as described.[11] $CD16^+$ natural killer (NK) cells and $CD4^+TCRV\beta8^+$ and $CD8^+TCRV\beta1^+$

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T cells were separated from peripheral blood mononuclear cells (PBMCs) by an EPICS Elite flow cytometer (Beckman Coulter Fullerton, CA). Approval was obtained from the human research committee of Kanazawa University Graduate School of Medical Science for these studies, and informed consent was provided according to the Declaration of Helsinki. Mutation analysis of *RAG* genes, fluorescence-activated cell sorter (FACS) analysis of TCRVβ repertoire, and complementarity-determining region 3 (CDR3) spectratyping were performed as described.[12,13]

Results and discussion

Inherited mutations in either the *RAG1* or the *RAG2* gene resulting in partial V(D)J recombination activity have been detected in most OS patients.[14] We found that our patient is homozygous for a single base C deletion after nucleotide 2113 of the *RAG1* gene (delC) in DNA from his granulocytes (Figure 1A). His parents were both heterozygous for this novel mutation. In contrast, DNA from the patient's PBMCs showed coexistence of the delC and other unexpected sequences (Figure 1A). When we analyzed such sequences in subcloned polymerase chain reaction (PCR) products obtained from his T cells, 6 different second-site mutations (mut no. 1–mut no. 6) were detected in addition to the delC mutation (Figure 1B). All of them restored the *RAG1* reading frame and resulted in missense mutations, which were located in the RAG2-interacting domain (Figure 1C). Sequencing analysis in the general population excluded the possibility that they could be functional polymorphisms. The possibility that his T cells were derived from the maternal T-cell engraftment was ruled out by fluorescence in situ hybridization analysis for the detection of the X/Y chromosome and by standard molecular study of HLA typing (data not shown). In addition, the second-site mutations were not detectable in the mother's PBMCs. We therefore concluded that T cells carrying the second-site mutations originated from the patient's own hematopoietic cells in vivo.

The incidence of revertant mosaicism is considered rare, and revertant cells have been shown to carry a single revertant sequence in reported cases.[6,7] Our studies, however, provide evidence for the presence of multiple and different second-site mutations in a single

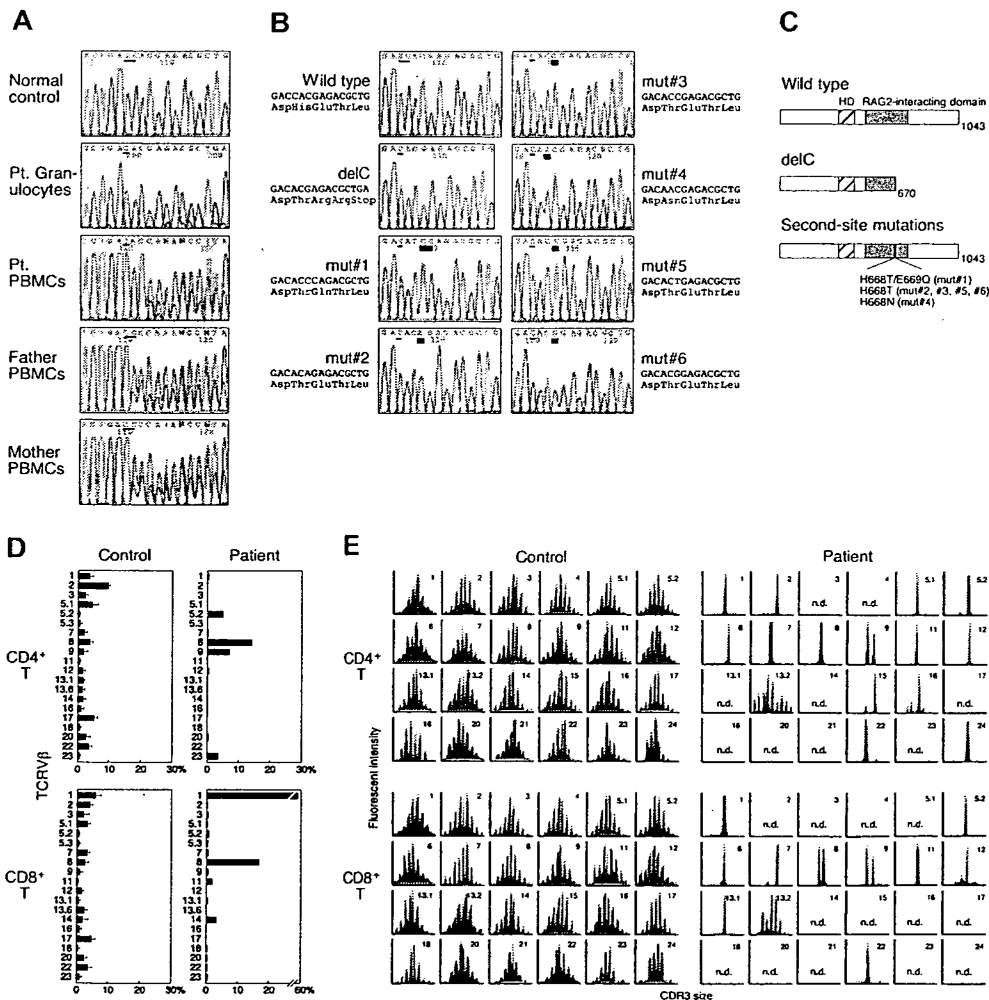

Figure 1. Characterization of *RAG1* gene mutations and T-cell receptor (TCR) Vβ repertoire. (A) The *RAG1* gene was amplified from DNA extracted from normal PBMCs, the patient's granulocytes and PBMCs, and the parents' PBMCs. Direct sequencing was performed using an automated sequencer. A thin bar shows the position of the delC mutation. Pt indicates patient. (B) Sequence analysis of the same genomic region in subcloned PCR products obtained from the patient's T cells. A thick bar highlights the position of the second-site mutations. (C) Predicted structures of mutated RAG1 molecules. HD indicates homeodomain. (D) Expression profile of TCRVβ subfamilies. Peripheral blood samples were stained with monoclonal antibodies (mAbs) for individual TCRVβ together with anti-CD4 and anti-CD8 mAbs. The percentage of each TCRVβ expression within CD4+ or CD8+ T cells was analyzed by a flow cytometry. (E) CDR3 spectratyping. Each TCRVβ fragment was amplified from cDNA with one of the Vβ-specific primers. The size distribution of PCR products was determined by an automated sequencer and GeneScan software.

Table 1. Genotypic analysis of lymphocyte subsets

			Second-site mutations					
	No.	delC	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
PBMCs	81	52	25	1	0	3	0	0
$CD4^+$ T cells	168	91	30	0	19	12	7	9
$CD8^+$ T cells	130	63	43	13	7	4	0	0
$CD4^+V\beta8^+$ T cells	45	29	0	0	16	0	0	0
$CD8^+V\beta1^+$ T cells	43	21	21	0	1	0	0	0
$CD16^-$ NK cells	13	13	0	0	0	0	0	0
Monocytes	23	23	0	0	0	0	0	0
Granulocytes	24	24	0	0	0	0	0	0

Sequence occurrence/total number of sequences.

patient with nonmalignant diseases, an occurrence previously unreported. The mechanisms underlying these findings are presently unclear. Although mutational hotspots such as repeat sequences or CpG dinucleotides or increased genomic instability could be responsible for an increased rate of reversion events,[6,7] this is unlikely the case in our patient.

The second-site mutations were only detectable among T lymphocytes including both $CD4^+$ and $CD8^+$ T cells and not among granulocytes, monocytes, and NK cells (Table 1). B lymphocytes were absent from his peripheral blood. These results suggest that the reversion events occurred in committed T-cell progenitors on one allele in the patient. Alternatively, some of the second-site mutations may have happened in more primitive hematopoietic progenitors such as common lymphoid progenitors, and the lack of circulating B cells, which is usually seen in typical OS patients, could be interpreted as the simple result of partial correction of V(D)J recombination activity. In OS, however, leaky differentiation of a very limited number of B cells is functional and results in the augmented IgE production. In contrast, serum IgE is usually undetectable in T^-B^- SCID with complete RAG deficiency, reflecting the impaired B-cell differentiation.[14] Therefore, it is suggested that no revertant event occurred within B-cell lineages in this patient. On the other hand, some mutants such as the mut no. 2, mut no. 5, and mut no. 6 were detected only in $CD4^+$ or $CD8^+$ T cells (Table 1), indicating that these second-site mutations may have occurred in T-cell progenitors at a stage after CD4/CD8 lineage commitment. Studies of lymphocyte development from *RAG1* and *RAG2* knock-out mice, however, have demonstrated that RAG-deficient thymocytes accumulate as quiescent cells with a heat-stable antigen (HSA)–positive, $CD25^+$, $CD4^-$, c-kit^{lo} phenotype resembling normal cells just prior to functional TCRβ chain expression.[15-17] These findings suggest that the mut no. 2, mut no. 5, and mut no. 6 might be derived from the other precedent mutants by second somatic events after CD4/CD8 lineage commitment.

RAG mutations lead to heterogeneous immune and clinical manifestations ranging from T^-B^- SCID to OS probably due to residual recombination activity.[14] The frameshift mutation delC is expected to abrogate RAG1 function and should have resulted in a T^-B^- SCID phenotype when present on both alleles. However, our patient is classified as OS based on the clinical findings. Although we need to perform V(D)J recombination assay to determine the restored activity, all of the patient's second-site mutations are likely compatible with partial correction of the RAG1 activity. Indeed, a similar missense mutation resulting in an E669G substitution has been reported in a patient with typical OS.[14] Accordingly, the revertant T cells of our patient showed mature and activated phenotype with a highly restricted TCR repertoire in the periphery (Figure 1D-E). It seems therefore reasonable that his clinical phenotype has changed from T^-B^- SCID to OS due to the revertant mosaicism.

Our studies provide significant implications of revertant mosaicism in the pathogenesis of OS. Recent advances in molecular genetics and cell enrichment techniques have allowed small levels of somatic mosaicism to be investigated.[18] Thus, somatic revertant mosaicism may play a more important role in factors that influence phenotypic expression of diseases than previously thought and will help us to understand, at least in part, inconsistent genotype/phenotype correlation in genetic disorders.

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Leaky phenotype of X-linked agammaglobulinaemia in a Japanese family

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Summary

X-linked agammaglobulinaemia (XLA) is an inherited immunodeficiency that is caused by a block in early B-cell differentiation. Whereas early B precursors in the bone marrow are present in substantial numbers, XLA-affected individuals have dramatically reduced numbers of circulating mature B cells, plasma cells and immunoglobulins of all isotypes. We report on a Japanese family with 3 XLA patients, in whom the serum immunoglobulin levels and number of B cells showed a significant difference among them in spite of harbouring the same splice donor site mutation in the BTK gene. We developed concise method for detection of this mutation, which is helpful for discovering the carrier. Patient 2 showed a significant serum immunoglobulin levels of all isotypes, including allergen-specific IgE. Expression of a normal and truncated size BTK gene was detected in patient 2's peripheral blood mononuclear cells (PBMCs). Expression of BTK protein was also detected in some B cells. These results suggest that the leaky phenotype in patient 2 was caused in part by the expression of a normal BTK gene transcript. The increased frequency of infection with age expanded the number of B cells with normal BTK gene expression and produced the serum immunoglobulin, including IgE.

Keywords: XLA, BTK gene, leaky phenotype, splice mutation, IgE production

Introduction

X-linked agammaglobulinaemia (XLA) is a rare genetic disorder of B-cell maturation characterized by the absence of mature B cells, very low serum levels of all immunoglobulin isotypes, and a lack of specific antibody production. Mutations in the gene coding for a tyrosine kinase (BTK, Bruton tyrosine kinase) have been identified as responsible for XLA but the exact role of this kinase in B cell development has not yet been established [1–5].

It is known that there was wide variability in a clinical presentation, even among the members of one family who are likely to be carrying the same gene.

Phenotypic variation within a 3-generation family has been described previously [6], in which a 51-year-old man with recurrent sinusitis and sporadic pneumonia was confirmed to have a mutation in a premature stop codon in the BTK gene. Other factors, such as infection exposures, have been postulated as possible reasons for phenotypic variation.

We came across a Japanese family with 3 X-linked agammaglobulinaemia. patients, one of whom exhibited a leaky phenotype. The patient showed the significant serum level of IgG, IgM, IgA and IgE. The analysis of XLA in a large family is useful for studying the genotype/phenotype relationship and our PCR-based method of detecting the mutation is helpful for discovering carriers of a BTK gene mutation.

Materials and methods

All of the XLA patients were diagnosed as clinical features, immunological phenotype and BTK protein expression. Patient 1 was a 3-years-old boy who was introduced to our hospital since he suffered from recurrent pyoderma. After the beginning of immunoglobulin replacement therapy, no severe infection has been observed. Patients 2 and 3 are p55–2 and p55–1, respectively [7]. The XLA 1–3 patients have different mutations of the BTK gene in this family. BTK gene mutations in XLA 1 and 3 were 1235–1247deletion and 1885G to T, respectively [7]. Informed consent for gene analysis was obtained from the patients or their parents.

Specific IgE antibodies

Specific antibodies for house dust and Dermatophagoides were measured with a fluoroenzyme immunoassay by means

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of a Uni-Cap assay kit (Pharmacia, Uppsala, Sweden). A specific IgE level higher than 3·5 IU/ml was considered positive.

Amplification and electrophoresis of the BTK gene

Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden). RNA was prepared from PBMCs and cDNA was synthesized with MMTV reverse transcriptase. Genomic DNA from PBMCs was prepared using a Sepa Gene kit (Sanko Jyunyaku, Tokyo, Japan). PCR primers for genomic DNA are as previously described [8]. PCR consisted of 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The amplified DNA fragment was electrophoresed using 2% agarose gel or 20% acrylamide gel [9].

For the concise detection of the IVS11 + 3G→T mutation we used mismatch primers, which were introduced artificially into the MseI site. The underlined nucleotide was a mismatched nucleotide.

sense 5′-GGAGGTTTCATTGTCAGAGAC-3′
antisense 5′-GTCCTCAGGGCCTTGGAATAGTAGCATT-3′

Following PCR amplification, the PCR product was digested using MseI. DNA was electrophoresed using 4% agarose gel or 20% acrylamide gel [10].

RT-PCR primers for the detection of the expression of exon 11 are as follows.

sense 5′-GTATGAGTGGTATTCCAAAC-3′
antisense 5′-GTCCTTTGGATCAATTTCCCAT-3′

Sequencing of the BTK gene

The PCR fragment was subcloned into a T-vector (Novagen, Madison, WI, USA) and sequenced using a dye primer or a dye terminator method with an autosequencer (Applied Biosystems, Indianapolis, IN, USA). For a dye primer sequencing five independent colonies were picked and sequenced.

Flow cytometric analysis of BTK expression in B cells

Intracellular BTK staining of B cells was performed as described previously [11]. The stained cells were analysed with by means of a flow cytometer (EPICS Cytomics FC500; Beckman Coulter KK, Tokyo, Japan). BTK protein expression was analysed in CD 19 positive B cells.

Table 1. Immunological data of XLA patients with IVS11 + 3G→T and the other mutations of the BTK gene.

	Age (years)	IgG	IgM	IgA	IgE	CD3	CD19
Patient 1	3	422	<5	<5	2·6	86·6	0·4
Patient2	29	480	63	151	269	85·2	2·1
Patient 3†	10	63	<5	<5			
XLA 1	31	495	<5	<5	<0·3	88·9	0·1
XLA 2	31	459	<5	<5	<0·3	68·7	0·1
XLA 3	20	381	6	<5	<0·3	89·0	0·1

†The serum immunoglobulin level except patient 3 is measured just before the immunoglobulin infusion. Patient 3's data is at 4 years old.

Results and discussion

Patient 1 was introduced to our hospital because of recurrent skin infection. His family has at least 3 XLA patients (Fig. 1). As shown in Table 1, patient 1 showed an extremely low serum immunoglobulin level in IgG, IgM, IgA and IgE (Patient 1's initial Ig levels before immunoglobulin replacement were as follows; IgG 136 mg/dl, IgA under 5 mg/dl, IgM 10 mg/dl, IgE 2·5 IU/ml). However, patient 2 showed detectable IgG, IgM and IgA. In his PBMCs CD19-positive B cells were also detected. Interestingly, IgE and allergen-specific IgE for house dust and mites was detected. Typical XLA patients (XLA 1–3) have no serum IgE (Table 1). It is assumed that IgE was the critical marker for the detection of the leaky phenotype.

Genomic DNA of the BTK gene in patient 1 revealed IVS11 + 3G→T resulting in the exon 11 skip. For the concise detection of the IVS11 + 3G→T mutation we used mismatch primers, which were introduced artificially into the MseI site. The mutant-type allele was digested into 73-bp and 29-bp fragments using MseI (Fig. 2), which indicated that the mother had both normal and mutant allele.

Expression of the BTK gene in patient 1 showed 80-bp-deleted transcripts and larger size transcripts compared with the normal size transcripts (Fig. 3a). Patient 1's mother

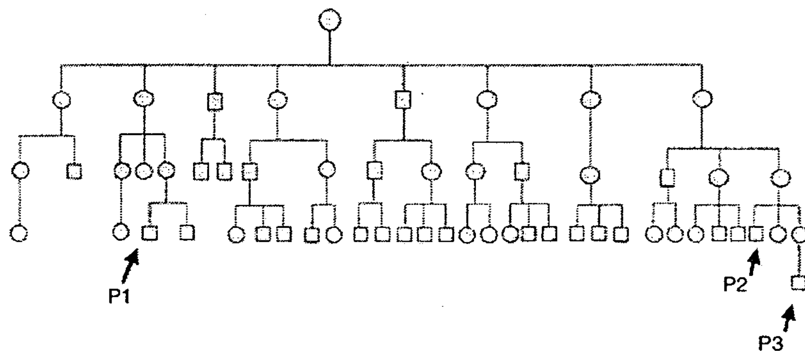

Fig. 1. The family tree of a large XLA family. The spouses in all cases were omitted. P1-P3 represent XLA patients 1–3 in Table 1, respectively.

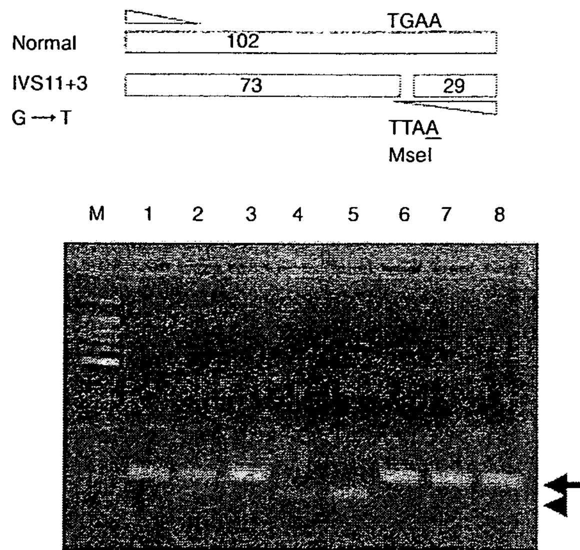

Fig. 2. The concise detection of the BTK gene mutation of IVS11 + 3G→T in this family. The upper panel shows the strategy for the detection of the mutation. An antisense strand mismatch primer, which introduced a MseI site de novo in the mutant sequence (underlined A), was used. The arrow indicates a band corresponding to normal DNA. The arrowhead indicates a MseI-digested band of a mutant DNA. M: size marker of DNA, lane 1: father of patient 1; lane 2: mother of patient 1; lane 3: elder brother of patient 1; lane 4: patient 1; lane 5: patient 2; lane 6–8: normal control.

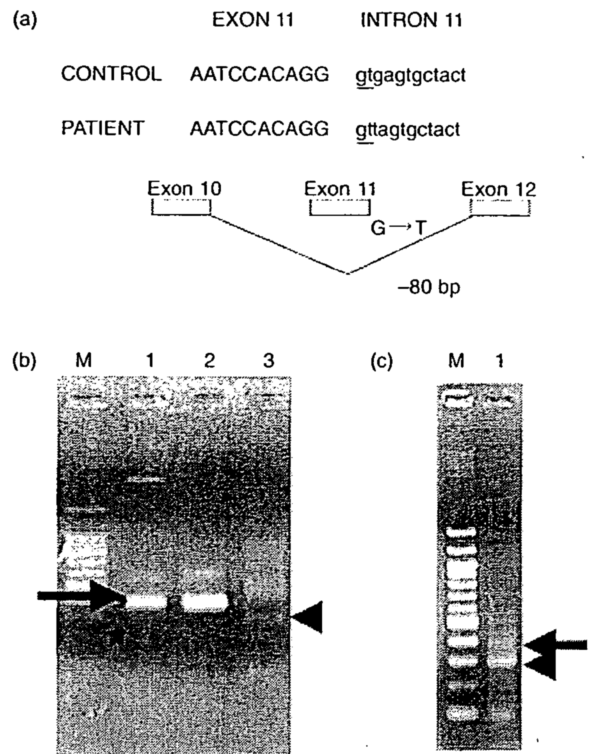

Fig. 3. The detection of normal size BTK gene expression in patient 2 but not in patient 1. (a) The abnormally smaller size PCR product in (b) and (c) was sequenced and found to be the result of a splicing abnormality caused by the mutation of intron 11. (b) The abnormally small size PCR product in the family of patient 1. The arrow indicates the normal size PCR product detected in the mother. The arrowhead indicates an abnormally small size PCR product. M: size marker; lane 1: mother of patient 1; lane 2: father of patient 1; lane 3: patient 1. (c) The abnormally small size and normal size PCR product in patient 2. The arrow indicates the normal size PCR product. The arrowhead indicates abnormally small size PCR product. M: DNA size marker; lane 1: patient 2.

showed weak 80-bp-truncated transcripts (Fig. 3b). On the other hand, patient 2 showed an 80 bp-truncated-band and normal size BTK gene transcripts (Fig. 3c).

We speculated that a leaky phenotype in the patient was the result of the occurrence of normal BTK transcripts in some B cells having the BTK gene mutation causing the exon 11 skip, which might lead to the antigen-driven expansion of B cells with normal BTK protein. To further validate this point, we tried to demonstrate the presence of normal BTK protein in some B cells in the patient with a leaky phenotype. For this purpose, we used a flow cytometric analysis using a monoclonal anti-BTK antibody. Figure 4 shows that 31% of CD19 positive B cells expressed normal BTK protein in patient 2. These results suggest that patient 2's peripheral B cells consist of BTK protein negative and positive B cells. In the patient 2's monocyte BTK expression was not detected by a flow cytometry (data not shown).

It is assumed that B cells which have normal BTK transcripts proliferate due to antigen stimulation since it is considered that the leaky B cells have the normal response to the antigen and might differentiate into memory B cells [12]. The difference of the function between BTK protein negative and positive B cells in patient 2 remains to be elucidated.

The serum immunoglobulin levels of patient 2 are significantly different from his nephew and the other typical XLA patients. These results suggest that the leaky phenotype of XLA might depend on the age and frequency of infection. We developed gel analysis after enzyme digestion for the concise detection of IVS11 + 3G→T. The analysis of XLA in a large family is useful for studying the genotype/phenotype relationship and our PCR-based method of detecting the mutation is helpful for discovering the carrier.

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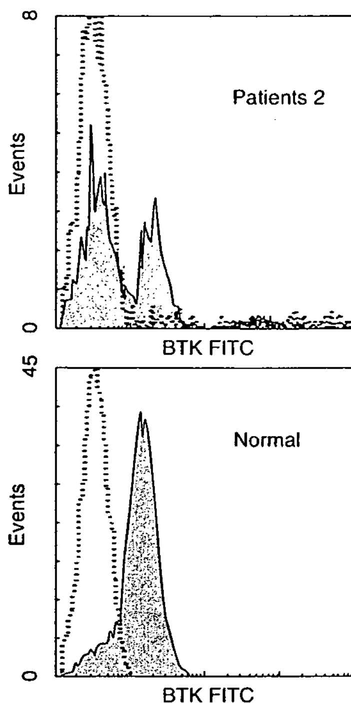

Fig. 4. Flow cytometric analysis of CD19 cell BTK expression in an normal donor and patient 2. The closed areas and dashed lines indicate the staining with anti-BTK and control IgG1 mouse antibodies in B cells, respectively.

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Analysis of class switch recombination and somatic hypermutation in patients affected with autosomal dominant hyper-IgM syndrome type 2

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Abstract

Autosomal recessive form of hyper-IgM syndrome type 2 (AR-HIGM2) is secondary to mutations affecting both alleles of *AICDA* gene encoding activation-induced cytidine deaminase, characterized by defects of immunoglobulin class switch recombination (CSR) and somatic hypermutation (SHM) in most of the patients. We herein report the immunological phenotype of seven patients carrying a single heterozygous R190X mutation in *AICDA*. Variable defect in in vivo CSR inherited as an autosomal dominant (AD) trait strongly suggests that this heterozygous *AICDA* mutation causes HIGM (AD-HIGM2). In AD-HIGM2 B cells, CSR was consistently found impaired in vitro. However, in contrast to AR-HIGM2, the CSR-induced double-stranded DNA breaks in the switch region of IgM heavy chain gene were detected. The SHM frequency in V regions of IgM heavy chain gene in B cells was normal in all (but one patient). The characteristics of the AD-HIGM2 phenotype indicate that the AID C-terminal region may be involved in DNA repair machinery required for CSR.
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Keywords: Activation-induced cytidine deaminase (AID); Autosomal dominant HIGM2; Antibody maturation; Class switch recombination; Somatic hypermutation; Double-stranded DNA repair

Introduction

Hyper-IgM syndrome (HIGM) is a primary immunodeficiency disease characterized by normal or high serum IgM levels associated with little or no serum IgG, IgA, and IgE, indicating a defect in the class switch recombination (CSR) process [1]. HIGM is heterogeneous and several molecular defects causing this condition have been described. The X-linked form (HIGM1) is caused by mutations in the gene encoding the CD40 ligand (CD40L/CD154) [2–5]. A deficiency in CD40 results in an autosomal recessive (AR) form of HIGM (HIGM3) [6]. CD40L is expressed on activated helper T cells and its interaction with CD40, which is constitutively expressed on B cells, is required for the formation of germinal centers in secondary lymphoid organs, B cell proliferation, CSR, and generation of somatic hypermutation (SHM) in the variable region of Ig.

HIGM due to an intrinsic B cell defect has also been reported [7,8]. One type of this HIGM condition displaying autosomal recessive inheritance (AR-HIGM2) is caused by mutations in the *AICDA* gene encoding the activation-induced cytidine deaminase (AID) [9]. AID is selectively expressed in B cells undergoing CSR and SHM in germinal centers [10]. The resulting defect in AID severely impairs both CSR and SHM. This finding, together with the HIGM phenotype of AID$^{-/-}$ mice [11], provides insight into the molecular mechanism involved in the secondary genetic modifications underlying B-cell terminal maturation within the germinal centers.

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Because of the similarity of its amino acid sequence to that of APOBEC-1 and its cytidine deaminase activity in vitro, AID was originally thought to be a mRNA-editing cytidine deaminase [10]. However, several observations provided evidence that AID acts as a single-stranded DNA (ssDNA)-specific deoxycytidine deaminase [12–16]. This model, in which AID deaminates cytosine to generate uracil residues on target DNA, is supported by the observation that uracil-DNA glycosylase (UNG) deficiency also leads to defective CSR in both mice and humans [17,18]. Indeed, UNG deglycosylates uracil residues produced on single-stranded DNA by AID activity, creating abasic sites that become targets for an apyrimidinic endonuclease resulting to single-stranded (ss) DNA breaks. It is unclear how these ssDNA breaks lead to the double-stranded DNA breaks (DSBs) necessary for CSR. Nevertheless, the CSR defect observed in AID and UNG deficiencies appears to be secondary to the defective generation of CSR-induced DSBs in the switch region of the IgM heavy chain gene (Sμ) [18–20]. The role of AID in the generation of SHM is unclear, but it has been suggested that AID acts differently in CSR and SHM [21,22]. Deoxyuracil induced by AID can be recognized either by UNG leading to the base excision repair or by mismatch repair system [23–25]. Either system leads to ssDNA breaks which can be repaired in an error-prone manner or which may be processed into DSB before repair. Alternatively, recent studies have suggested that blunt-ended DSBs occur spontaneously in V regions in an AID-independent manner [20,26–28]. AID may secondarily transform blunt-ended DSBs into DSBs with overhanging ends, which are repaired by error-prone polymerases [28].

To date, 39 different mutations in the *AICDA* gene have been identified in 75 AR-HIGM2 patients, mostly in the homozygous state, but in some cases as compound heterozygous mutations (Refs. [9,29–31] and our unpublished data). We previously reported that mutations in *AICDA* gene affecting the C-terminal part of AID result in a CSR-specific defect with normal SHM [22]. Among these mutations, a peculiar one, R190X resulting in the predicted truncation of the 9 last amino acids of the nuclear export signal (NES) of AID, appears to exert a dominant-negative effect because heterozygous patients suffer from HIGM of variable intensity [22,32]. This mutant was shown to exert no CSR activity when expressed in AID-deficient murine splenic B cells while SHMs were induced on an artificial substrate after transfection of R190X in fibroblasts [22]. A similar discrepancy was observed in in vitro experiments using an artificial mutant leading to the truncation of the last 10 amino acids of AID [33]. When expressed in AID-deficient murine splenic B cells, this mutant was defective for CSR while it induced gene conversion and Vλ SHM when transfected in the AID-deficient avian B cell line DT40. Despite complete lack of CSR, mutations in the Sμ region were normally observed, likely ruling out a defect of AID targeting to Sμ regions [33]. The effect of heterozygous R190X mutant was related to the loss of functional NES since this mutant, when transfected in fibroblasts, accumulates into the nucleus, likely overriding the normal allele expression [34].

Because these data have been obtained in in vitro experiments (over-expression in *E. coli*, chicken cell line, or mouse B cells), we attempted to get insight into the physiological relevance of the C terminal part of AID by characterization of the immune defect of $AID^{R190X/+}$ patients. We studied 7 patients from 3 unrelated families and found a variable HIGM phenotype transmitted as an autosomal dominant trait. We provide evidence that the CSR defect, which was drastic in vitro, occurred downstream from the double-stranded DNA breaks. In all, but one patient, the frequency of SHM was found normal. Our results suggest thus that AID, besides its cytidine deaminase activity, plays a role by possibly recruiting to its C-terminal domain a co-factor involved in CSR-DNA repair.

Patients, materials and methods

Patients

Seven patients from three unrelated non-consanguineous families (Fig. 1) were diagnosed as affected with HIGM on the basis of low serum IgG, IgA, and IgE concentrations and high serum IgM concentrations (Table 1). Blood counts of B cells ($CD19^+$), including $CD27^+$ B cells, and T cells ($CD3^+$, $CD4^+$, $CD8^+$) were similar to those of normal controls (data not shown). Immunoglobulin levels of the patient 2-I-1 was reported previously as JP42 [22].

This study was carried out in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee. Informed consent was obtained from all patients or their parents before the study.

AICDA gene sequencing

Genomic DNA was extracted from the granulocytes of the patients with Sepa Gene (Sanko Junyaku, Tokyo, Japan). The five exons and adjacent intron sequences of *AICDA* were amplified by PCR with the high fidelity *Taq* polymerase (GibcoBRL) and sequenced [9].

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) after stimulation with soluble CD40 ligand (sCD40L, 500 ng/ml, kindly donated by Amgen, Seattle WA, USA) and recombinant interleukin-4 (rIL-4, 100 U/ml, R&D Systems, Minneapolis MN, USA) for 5 days or from Epstein–Barr virus-transformed lymphoblastoid B-cell lines (EBV-LCLs) using the Trizol reagent (Invitrogen BV, Groningen, Netherlands). The first-strand cDNA was produced from 2 μg of total RNA with the

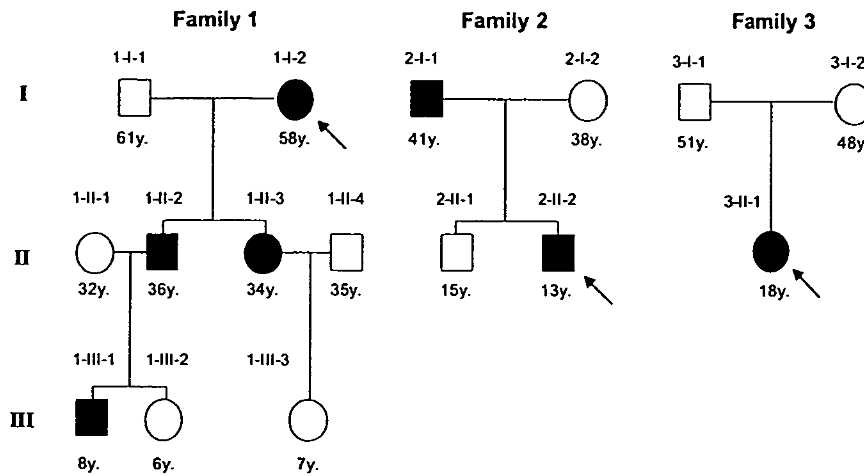

Fig. 1. Three unrelated families affected by an autosomal dominant hyper-IgM syndrome. Closed symbols indicate patients with hyper-IgM syndrome (HIGM) with a heterozygous R190X mutation of the *AICDA* gene. Open symbols indicate members of the families without HIGM and *AICDA* mutation. Age (years) at the time of the study is indicated. Arrows indicate the first patient diagnosed in each family.

ThermoScript RT-PCR System (GibcoBRL) using oligo dT as a primer. The *AICDA* cDNA was then amplified by PCR with the forward primer 5′-GAGGCAAGAAG-ACACTCTGG-3′ and the reverse primer 5′-GTGACATT-CCTGGAAGTTGC-3′ (94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a final extension for 7 min at 72°C). The PCR products were directly sequenced and analyzed using the Big Dye Terminator DNA sequencing kit (Perkin-Elmer Life Sciences), internal primers and an automated ABI PRISM 310 genetic analyzer (Applied Biosystems), as previously described [9].

We assessed the level of expression of *AICDA* transcripts from the two alleles by carrying out PCR amplification of exon 5 with a sense mismatched primer introducing an *Nde*I site into the c.568 C > T allele (5′-CCTGTATGAGGTT-GATGACATA-3′) and a matched antisense primer (5′-CTGTCTTCAGAGATATT-3′). PCR products were subjected to *Nde*I digestion followed by electrophoresis in a 4% agarose gel.

Table 1
Clinical findings in patients

Individual	Age at diagnosis (years)	Recurrent infections	IgM (mg/dl)	IgG (mg/dl)	IgA (mg/dl)	IgE (IU/ml)	AID
1-I-1	61	–	228	1893	215	98	Normal
1-I-2	58	+	405	128	10	<5	R190X/Normal
1-II-1	32	–	153	1548	164	290	Normal
1-II-2	36	–	285	346	51	<5	R190X/Normal
1-II-3	34	–	302	633	39	<5	R190X/Normal
1-II-4	35	–	128	1364	164	350	Normal
1-III-1	8	–	122	758	45	<5	R190X/Normal
1-III-2	6	–	95	894	63	12	Normal
1-III-3	7	–	175	1267	148	19	Normal
2-I-1	41	+	293	282	220	<5	R190X/Normal
2-I-2	38	–	243	1691	197	483	Normal
2-II-1	15	–	164	1295	157	400	Normal
2-II-2	13	+	234	21	<5	<5	R190X/Normal
3-I-1	51	–	239	1354	253	23	Normal
3-I-2	48	–	201	1548	156	308	Normal
3-II-1	18	+	234	38	<5	<5	R190X/Normal
$AID^{+/+}$(n = 9)	33 ± 20	0/9	181 ± 51	1428 ± 286	169 ± 52	220 ± 183	mean ± SD
$AID^{R190X/+}$(n = 7)	30 ± 18	4/7	268 ± 86*	315 ± 288***	54 ± 76**	0 ± 0*	
$AID^{-/-}$(n = 13)	31 ± 16	13/13	2744 ± 1833***	4 ± 16***	0 ± 0***	0 ± 0**	
$AID^{+/-}$(n = 5)		0/5	144 ± 63	1277 ± 294	153 ± 73	115 ± 103	
Normal range	3–8 years		50–118	680–1260	66–162	–	
	Adult		40–230	700–1600	70–400	<170	

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ compared with $AID^{+/+}$.

B-cell proliferation and IgE production

Peripheral blood mononuclear cells (PBMCs) were isolated and incubated with soluble CD40L (500 ng/ml) and rIL-4 (100 U/ml, R&D Systems) for 5 days for proliferation assays and for 12 days for IgE production. Proliferation was assessed by measuring [^{3}H] thymidine uptake. Total RNA was extracted from PBMC after 5 days of activation by sCD40L and rIL-4 by Trizol (Invitrogen) and cDNA was produced using reverse transcriptase (Superscript II, Invitrogen) with an oligo dT primer (Invitrogen) according to the manufacturer's instructions. *CD19*, *AICDA*, germ line (GLTε), and functional transcripts (FTε) of IgE were detected by RT-PCR as previously described [7,9]. IgE production in culture supernatants was determined by ELISA as previously described [35]. All values were determined in triplicate. Healthy family members (AID$^{+/+}$) and parents carrying heterozygous mutations of the *AICDA* gene affecting the cytidine deaminase domain (W68X), the APOBEC-1 like domain (R174S), or consisting of a deletion of the entire coding region were also analyzed (AID$^{+/-}$).

CSR-induced double-stranded DNA breaks

DSBs in the Sμ region were detected by means of a ligation-mediated PCR method, as previously described [20,36]. PBMCs were activated by incubation with sCD40L and IL-4 for 5 days, and PI$^-$ (viable) CD19$^+$ (B) cells were then selected by sorting with an FITC-conjugated anti-CD19 monoclonal antibody (Immunotech, Marseilles, France) and propidium iodide (Sigma-Aldrich, Taufkirchen, Germany). Genomic DNA was extracted from viable B cells (>94% pure) in 1% low melting agarose (Gibco) overlaid with DNA extraction buffer (50 mM Tris, 20 mM EDTA, 1% sodium laurylsarcosyl, 10 mM protein K). A hemi-double-stranded linker (Bw) was ligated to DNA using T4 DNA ligase (Promega, Madison WI, USA). Semi-nested PCR was performed with Sμ region-specific primers (Sμext: 5′-ATGGAAGCCAGCCTGGCTGT-3′ and Sμint: 5′-AGCCTGGCTGTGCAGGAACC-3′) and a linker-specific primer. The PCR products were separated by electrophoresis, transferred to a membrane (Genescreen Plus, Perkin-Elmer Life Sciences, Boston MA, USA), and hybridized with a ^{32}Pγ-ATP (Amersham Bioscience, Uppsala, Sweden)-labeled gene-specific oligonucleotide probe recognizing the Sμ region (Sμ probe: 5′-TCAGAAATGGACTCAGATGG-3′).

Analysis of somatic hypermutation in variable regions of the immunoglobulin gene

Total RNA was extracted from unstimulated PBMCs and cDNA generated as previously described [30]. The VH5-51 region of the Ig gene was amplified by PCR with primers corresponding to the 5′ region of the VH5 leader sequence 5′-ATGGGGTCAACCGCCATCCT-3′ and to the constant region of the IgM heavy chain (Cμ) 5′-GTCCTGTGCGAGGCAGCCAA-3′. PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, with a final 7-min extension period at 72°C. The enzyme used was Platinum High Fidelity *Taq* DNA polymerase (Invitrogen). The PCR products were subsequently cloned into pGEM-T Easy Vector System II (Promega), and at least eight clones were sequenced with the BigDye Terminator Cycle Sequencing Kit (ABI PRISM) and analyzed with and ABI PRISM 310 Genetic Analyzer.

SHM generation in the VH3-23 region in CD19$^+$CD27$^+$ B cells was also assessed in some patients as previously described [9]. PBMCs were purified by flow cytometry using FITC-anti-CD19 and PE-anti-CD27 monoclonal antibodies (Immunotech). Total RNA was purified with the Trizol reagent and cDNA was obtained by reverse transcription with an oligo dT primer. PCR was carried out with the Pfu polymerase (PfuTurbo, Stratagene) using primers for the VH3-23 leader exon (5′-GGCTGAGCTGGCTTTTTCTTGTGG-3′) and Cμ region (CμB; 5′-TCACAGGAGACGAGGGGGAA-3′) (35 cycles at 94°C for 45 s, 60°C for 1.5 min, 72°C for 2 min). PCR products were subcloned and analyzed as described above.

Results

An autosomal dominant hyper-IgM syndrome is associated with a heterozygous mutation of the AICDA gene generating a premature stop codon (AD-HIGM2)

We have studied seven patients with HIGM from three unrelated families from Japan (Fig. 1). In all patients, serum IgM concentration was high or normal whereas serum IgG and/or IgA concentrations were decreased (Table 1). One patient (1-III-1) had a normal IgG concentration (758 mg/dl) and a low IgA concentration (45 mg/dl) while another patient (2-I-1) had a normal serum IgA concentration (220 mg/dl) and a low IgG concentration (282 mg/dl).

In two families (families 1 and 2), the HIGM phenotype was found in several generations (three in family 1 and two in family 2), in both male and female subjects, suggesting an autosomal dominant transmission. In family 1, patient 1-I-2 had recurrent pneumonitis and bronchitis beginning at the age of 43 years. HIGM was diagnosed in this patient at the age of 53 and intravenous immunoglobulin (IVIG) treatment started 1 year later. No other family member had obvious episodes of recurrent infection. Patients 1-II-2, 1-II-3, 1-III-1 were diagnosed with HIGM following systematic investigation of serum Ig concentrations. None of the members of family 1, including patient 1-I-2, presented lymphoid organ hyperplasia or autoimmune diseases. In family 2, patient 2-II-2 was diagnosed with HIGM at the age of 13 years because of recurrent episodes of respiratory tract

infection, enlarged tonsils, and abnormal serum concentrations of IgG, IgA, and IgM. Examination of the tonsils revealed the characteristic enlarged germinal centers typically observed in AR-HIGM2 patients [9]. Patient's father (patient 2-I-2) also had recurrent respiratory tract infections and enlarged tonsils. No autoimmune diseases were observed. In family 3, patient 3-II-1 was diagnosed at 18 years of age on the basis of recurrent respiratory tract infections and abnormal serum Ig concentrations. Family members presenting normal serum Ig concentrations were healthy. CD40 and CD40L were normally expressed on patients' B cells and on PMA- and ionomycin-activated T cells, respectively (data not shown), and *CD40L* and *CD40* gene sequences were found to be normal.

Despite the fact that HIGM2 with *AICDA* gene mutations has been reported to be inherited in an autosomal recessive manner [9,29–31], we sequenced the *AICDA* gene in these patients. The same heterozygous nonsense mutation in exon 5 (c.568 C > T) of the *AICDA* gene was found in each case. This mutation changes Arg190 to a stop codon (R190X), truncating the AID molecule by removing the nine C-terminal amino acids in the nuclear export signal (NES) domain. No mutation was found in the other exons or in the adjacent intronic sequences and 5′ and 3′ non-coding flanking regions. This mutation was not found in 526 Japanese subjects [37]. All healthy members within the three families had normal *AICDA* gene sequences ($AID^{+/+}$).

The heterozygous nature of the mutation was confirmed by direct sequencing of cDNA obtained from sCD40L + IL-4-activated patients' PBMCs. We checked the expression of the mutated and unmutated alleles by amplifying the *AICDA* exon 5 cDNA by RT-PCR, introducing an *Nde*I restriction site into the c.568 C > T mutant by using a mismatch primer, as previously described [32]. After digestion with *Nde*I, unmutated (undigested) and mutated (digested) alleles were found to be both expressed in all patients tested (P1-I-2, P-1-II-3, P2-I-1, P2-II-2) (data not shown).

The finding of a heterozygous mutation in *AICDA* is consistent with the autosomal dominant inheritance as observed in families 1 and 2. Parents of patient 3-II-1 had normal *AICDA* genes and did not display an HIGM phenotype, suggesting that this patient had probably acquired a de novo mutation of the *AICDA* gene in one allele, as found in a previously reported patient [32]. All but one member of family 1 (patient 1-III-1) carrying the heterozygous R190X mutation of the *AICDA* gene ($AID^{R190X/+}$) had significantly lower concentrations of IgG, IgA, IgE and significantly higher concentrations of IgM than healthy $AID^{+/+}$ family members or subjects with other heterozygous *AICDA* mutations ($AID^{+/-}$, n = 5, W68X, 79–81 in frame deletion, R112H, R174S or deletion of the whole coding region of the *AICDA* gene). These data show that there is a correlation between the heterozygous R190X mutation and the HIGM phenotype. However, the Ig defect was less pronounced than the one found in $AID^{-/-}$ patients, except for patients 2-II-2 and 3-II-1 (Table 1).

The defect in class switch recombination occurs beyond the DNA cleavage step

As AD-HIGM2 patients display defects in CSR of various in vivo intensity, in vitro response to soluble CD40 ligand (sCD40L) and IL-4 activation of patients PBMC was tested. We consistently observed a normal proliferative response (data not shown) and normal expression of germline transcripts of the ε heavy chain gene (Fig. 2A). Expression of *AICDA* transcripts was well correlated with expression of *CD19* transcripts, giving evidence of the induction of *AICDA* in the patients' cells (Fig. 2A). On the other hand, no CSR towards IgE was observed, as shown by the lack of functional transcripts (Fig. 2A) and of IgE production (Fig. 2B). This defect contrasted sharply with the normal CSR observed in PBMCs from $AID^{+/-}$ subjects (n = 5, heterozygotes for W68X, R174S, or deletion of the whole coding region of the *AICDA* gene). It was, however, reminiscent of the complete lack of CSR observed in $AID^{-/-}$ patients [9]. (Fig. 2B).

In order to identify the stage at which CSR was defective, the formation of double-stranded DNA breaks (DSBs) in the switch region of the IgM heavy chain gene (Sμ) was analyzed by means of a ligation-mediated PCR (LM-PCR) technique and hybridization using a Sμ labeled probe (Fig. 2C) [20,36]. We found a similar occurrence of DSB in activated viable B cells from $AID^{R190X/+}$ patients and controls, suggesting that the CSR defect observed in patients occurred downstream from the DNA cleavage step (Fig. 2C). The sequencing of subcloned LM-PCR products revealed that DSB indeed occurred within the Sμ region (data not shown). This finding sharply contrasts with the absence or the dramatic decrease in the occurrence of CSR-induced DSB in the Sμ regions of activated B cells from $AID^{-/-}$ patients [20]. DSB observed in Sμ regions in activated B cells from $AID^{R190X/+}$ patients could have been induced by wild-type AID, although we cannot exclude that they reflect the high sensitivity of the technique used for DSB detection. However, no functional IgE transcripts were detected by RT-PCR, and IgE production was null in culture supernatants, suggesting an actual dominant negative effect exerted by the mutated form of AID.

SHM in AD-HIGM2 patients

Somatic hypermutation (SHM) of the variable (V) region of the immunoglobulin gene is another mechanism of antibody diversification which is dependent on AID activity [9,11,38]. However, it was recently reported that AID mutations affecting the C-terminal part of AID do not affect SHM generation [22,33]. We therefore analyzed SHM in the VH5-51 region in B cells from all $AID^{R190X/+}$ patients and healthy family members as described previously (Table 2A) [30]. In six out of the seven patients, the frequency of SHM was found normal (Fig. 3A). One patient (patient 2-II-2) displayed a very low frequency of SHM in the VH5-51

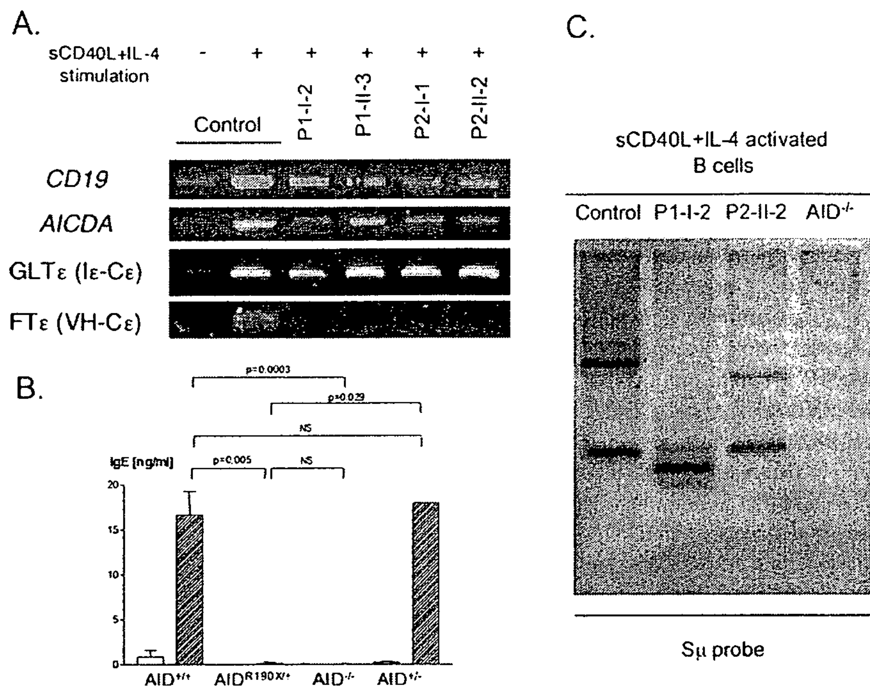

Fig. 2. In vitro CSR defect in $AID^{R190X/+}$ patients. (A) Normal induction of *AID* and germline transcripts of the *IgE* gene but impaired induction of functional transcripts of the *IgE* gene in patients' B cells after stimulation with soluble CD40 ligand and IL-4. Transcripts of the *CD19* (as control), *AICDA* genes, and germline (GLTε; Iε-Cε) and functional (FTε; VH-Cε) *IgE* gene transcripts were detected by RT-PCR after stimulation with soluble CD40 ligand (sCD40L) and IL-4 for 5 days in control subjects or in heterozygous R190X/+ patients (P1-I-2, P1-II-3, P2-I-1, P2-II-2). (B) Defective IgE production by patients' B cells after stimulation with soluble CD40 ligand and IL-4. PBMCs from normal controls and normal family members ($AID^{+/+}$, n = 14), HIGM patients with heterozygous R190X *AICDA* gene mutation ($AID^{R190X/+}$, P1-I-2, P1-II-3, P2-I-1, P2-II-2), hyper-IgM syndrome patients with homozygous or compound heterozygous mutations of *AICDA* gene ($AID^{-/-}$, n = 8) [9], or asymptomatic family members carrying heterozygous *AICDA* gene mutations other than R190X ($AID^{+/-}$, n = 5, W68X, R174S, or deletion of the entire coding region) were stimulated by incubation with soluble CD40 ligand (sCD40L) and IL-4 for 12 days. White bars: no stimulation. Gray bars: 12 days of stimulation with sCD40L and IL-4. Concentrations of IgE in the culture supernatants were quantified by ELISA. Error bars show the standard deviation. An unpaired t test was used to compare both groups. NS: not significant (P > 0.05). Similar results were obtained in $AID^{+/+}$ family members (n = 5) and all $AID^{R190X/+}$ patients (n = 7) using anti-CD40 mAb and IL-4 activation (data not shown). (C) Occurrence of CSR-induced DNA double-stranded breaks in the switch region of the IgM gene (Sμ) in patients' B cells after stimulation with soluble CD40L and IL4. PBMCs were stimulated with soluble CD40 ligand (sCD40L) and IL-4 for 5 days. Viable B (PI^-CD19^+) cells from control, $AID^{R190X/+}$ patients (P1-I-2, P2-II-2), and an $AID^{-/-}$ patient (compound heterozygote of W68X in one allele and deletion of the entire *AICDA* coding region in the other allele) sorted on a FACScan by using a FITC-conjugated anti-CD19 monoclonal antibody and propidium iodide. Genomic DNA was ligated using a partially double-stranded linker, and semi-nested PCR was performed with linker-specific and Sμ region-specific primers. PCR products were subjected to electrophoresis, transferred to a membrane, and hybridized with an Sμ region-specific probe labeled with γ^{32}P-ATP.

region (0.12%/bp, 33% of clones being mutated), although the number of $CD27^+$ B cells was found to be normal. To confirm these results, we checked the frequency of SHM in the VH3-23 region of purified $CD27^+$ B cells from four patients. In three cases (1-I-2, 1-II-3, 2-I-1), we found a SHM frequency similar to that of healthy individuals ($AID^{+/+}$) or

Table 2
Somatic hypermutation (SHM) in VH regions

	Clones			Mutations		Nucleotide changes			
	Mutated	Total	%	Total	Frequency [%bp]	GC target [%]	GC transition [%]	C > T mutation [%]	AT transition [%]
A. SHM in VH5-51 region in B cells									
R190X/+ (n = 7)	35	64	54.5	242	1.3 ± 0.8	71.8 ± 11.3	68.1 ± 7.2	23.4 ± 6.2	46.3 ± 16.1
Control (n = 7)	39	69	56.5	364	2.0 ± 1.5	68.1 ± 9.0	60.4 ± 8.0	21.1 ± 2.4	52.6 ± 6.4
B. SHM in VH3-23 region in $CD27^+$ B cells									
R190X/+ (n = 4)	29	31	93.5	398	3.6 ± 2.8	74.6 ± 3.4	66.6 ± 6.8	23.3 ± 2.6*	51.5 ± 5.8
Control (n = 8)	70	70	100	885	4.0 ± 1.3	64.3 ± 4.2	58.8 ± 7.0	11.7 ± 2.9	50.8 ± 9.8
+/– (n = 5)	29	30	96.7	407	4.2 ± 1.3	63.4 ± 3.9	57.4 ± 6.9	12.9 ± 3.6	60.1 ± 10.3

+/–: careers of autosomal recessive hyper-IgM syndrome type 2 with heterozygous *AICDA* gene mutations (either W68X, 79–81 deletion, R112H, R174S, or deletion of whole coding region).
* P = 0.0003.

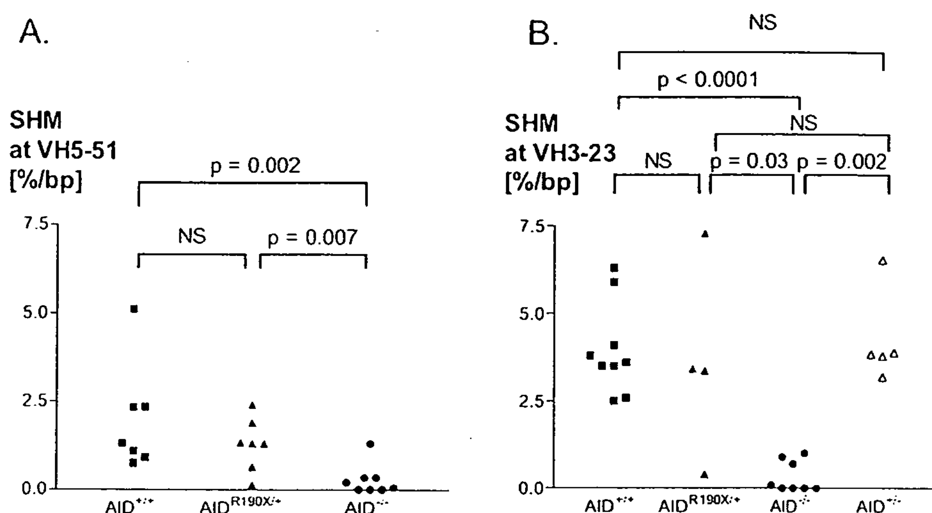

Fig. 3. Ig somatic hypermutation in B cells from $AID^{R190X/+}$ patients. Somatic hypermutation (SHM) of the VH5-51 (A) or VH3-23 (B) region of IgM in PBMC (A) or purified $CD19^+27^+$ B cells (B) from controls, including family members without *AICDA* gene mutation ($AID^{+/+}$, $n = 7$ in A and $n = 9$ in B), HIGM patients with heterozygous R190X *AICDA* gene mutation ($AID^{R190X/+}$, $n = 7$ in A and $n = 4$ in B), HIGM patients with homozygous or compound heterozygous *AICDA* gene mutations ($AID^{-/-}$, $n = 8$ [30] in A and $n = 8$ [9] in B), and asymptomatic parents of HIGM patients with homozygous or compound heterozygous *AICDA* gene mutations ($AID^{+/-}$, $n = 5$ in B, heterozygotes for W68X, R174S, or deletion of the entire *AICDA* coding region) was analyzed. An unpaired t test was used. NS: not significant ($P > 0.05$).

$AID^{+/-}$ subjects with heterozygous mutations of *AICDA* other than R190X (Table 2B). However, as observed in the VH5-51 region, the frequency of SHM was decreased in the VH3-23 region in patient 2-II-2 (0.4%/bp, 25% of clones being mutated) (Fig. 3B).

The nucleotide substitution patterns of SHM were also studied in $AID^{R190X/+}$ patients. Although no difference was observed in the VH5-51 region between $AID^{R190X/+}$ patients and controls (Table 2A), transition mutations at dC (dC > dT) were found significantly increased in VH3-23 region, as compared to $AID^{+/+}$ ($P = 0.0003$) or $AID^{+/-}$ ($P = 0.0054$), but transition mutations at dG (dG > dA) were found comparable to controls (Table 2B).

Discussion

We herein report the occurrence in seven patients of an autosomal dominant form of the HIGM condition caused by the same heterozygous *AICDA* mutation ($AID^{R190X/+}$). The latter is predicted to result in the C-terminal truncation of the AID protein, with a loss of the last nine amino acids in the NES domain. This heterozygous mutation was previously described by us [22] and another group [32], but no analyses of CSR and SHM in patients' B cells were so far reported. The aim of our study was the precise definition of the phenotype of AD-HIGM2. Four of the seven patients had experienced recurrent infections. None of the patients had an autoimmune disease, whereas 21% of patients with typical AR-HIGM2 do present such manifestations [31]. Clinical phenotype and changes in serum Ig concentrations indicated a mild phenotype as compared to AR-HIGM2 patients, with the exception of patients 2-II-2 and 3-II-1. The frequency of SHM was normal in all but one patient (P2-II-2). Strikingly, this patient displayed severely impaired CSR and SHM, as observed in $AID^{-/-}$ patients. His tonsil biopsy also revealed the giant germinal centers characteristic of $AID^{-/-}$ patients. However, we could not give evidence for a biased expression of the mutated *AICDA* allele. The variable penetrance of AD-HIGM2 has yet to be accounted for, but other genetic and/or environmental factors could also be involved.

Although serum IgG and IgA concentrations were affected to a variable magnitude, B cells from all heterozygous $AID^{R190X/+}$ patients displayed a similar profound defect in in vitro CSR. In contrast, the frequency of SHM in the variable regions of the Ig gene was found normal in six out of the seven patients. These results are consistent with the results of recently reported experiments using *AICDA* mutants transfected into either AID-deficient murine splenic B cells or DT40 chicken B-cell line or fibroblasts, demonstrating that the C-terminal domain of AID is required for CSR, but is dispensable for SHM and gene conversion [22,33]. In *E. coli*, the DNA cytidine deaminase activity of C-terminally truncated AID is detectable and even elevated as compared to wild-type [33]. These results strongly suggest that AID not only induces CSR through its cytidine deaminase activity, but also plays a second role via potential interaction of its C-terminal domain with a CSR-specific cofactor [22]. This cofactor may be required either for AID targeting on S regions or for CSR-induced DSB repair. Two observations suggest that the latter hypothesis is more likely, (i) S targeting has been shown to occur in the absence of the C-terminal part of AID in mice, since mutations in Sμ regions could be detected in the expected frequency [33] and (ii) the CSR defect observed in

AID$^{R190X/+}$ patients occurs downstream from Sμ region DNA cleavage since DSBs were detected in Sμ regions of CSR-activated B cells. Strikingly, the AD-HIGM2 phenotype is very similar to that of HIGM4, i.e., defective CSR with normal DSB occurrence and normal SHM frequency, thought to be caused by a CSR-associated DNA repair defect [36]. Transition mutations on C residues (dC > dT) were significantly increased in the VH3-23 region of CD27^{+} B cells from 3 patients, an observation not found in VH5-51 region. This difference, if significant, could suggest an unexpected function of the C terminal part of AID in SHM, which cannot be detected on artificial SHM substrates. Indeed, it has been recently shown that the RPA2, molecule already known to bind UNG, also binds to phosphorylated AID, facilitating its targeting on RGYW motifs and its cytidine deamination on the DNA non-template strand (leading to dC > dT after replication) [39]. The differences of dC transitions in VH5-51 and VH3-23 regions might reflect a difference in their respective RPA requirement, especially because these two regions differ in their respective RGYW motif content (13 motifs in VH3-23 and 8 motifs in VH5-51).

The HIGM condition associated with heterozygous *AICDA* R190X mutation observed in three families is unlikely to be accounted for by AID haploinsufficiency since it is not found in subjects carrying heterozygous mutations of the *AICDA* gene affecting the cytidine deaminase and APOBEC-1-like C-terminal domains or involving deletion of an entire *AICDA* allele. A dominant negative effect could be accounted for the formation of abnormal AID dimers or tetramers. Indeed, AID has been shown to form homodimer or homotetramer [16,22], in a C-terminal domain independent way [22]. Multimeric AID could interact with CSR-specific co-factor binding to its C-terminal domain. Conversely, according to the model based on the structure of yeast RNA cytidine deaminase (ScCDD1), the C-terminal part could be important for the multimeric AID binding to the phosphate backbone of target DNA. Thus, an impairment of AID binding to DNA could lead to defective CSR, but this hypothesis does not account for the CSR-induced DSB occurrence observed in AID$^{R190X/+}$ B cells. Another hypothesis is that the AD-HIGM phenotype is caused by the nuclear accumulation of the mutated form of AID overriding the expression of the normal AID allele since the NES domain truncation impairs nuclear export of AID [34,40,41].

That a heterozygous *AICDA* mutation resulting in the C-terminal truncation of AID is associated with the AD-HIGM2 condition suggests that AID could exert a cytidine-deaminase-independent function by binding to a molecular complex involved in CSR [42]. Identification of these putative AID co-factors would increase our understanding of the precise role of AID in antibody maturation events and should contribute to the definition of other HIGM syndromes.

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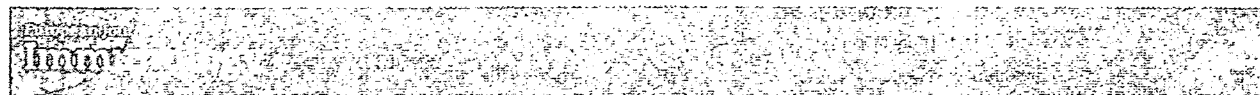

CD72-mediated suppression of human naive B cell differentiation by down-regulating X-box binding protein 1

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B cells can differentiate into antibody-secreting plasma cells, however the signals that control the entry into this pathway are not clearly understood. We have investigated the role of human CD72 in mature B cell differentiation. Human CD72 is preferentially expressed in naive B cells, but marginal levels of expression can be found in switched memory B cells. CD72 cross-linking promoted an increase in B cell activation and proliferation. Interestingly, expression of CD27, whose signal induces the differentiation of B cells into plasma cells, was down-modulated by CD72 stimulation. This CD72 signaling also induced tyrosine phosphorylation of various proteins such as Blk. Plasma cell differentiation and Ig syntheses were diminished by CD72 ligation in the presence of *Staphylococcus aureus* Cowan strain (SAC) plus IL-2 but not in the presence of CD40 signaling or CpG oligodeoxynucleotide. Our results show that CD72 signaling reduces the expression of X-box binding protein 1 in B cells stimulated with SAC plus IL-2, but the expression of PRDI-BF1 was unaffected. Taken together, these data demonstrate that CD72 is a key molecule in regulating mature B cell differentiation, particularly in preventing the differentiation of naive B cells into plasma cells, thus blocking the production of low-affinity antibodies.

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Introduction

Various surface proteins on B cells regulate B cell signaling and positive or negative responsiveness. Among them, several molecules such as paired Ig-like receptor PIR-B, FcγRIIB (CD32), CD22 and CD72 [1, 2] contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails. They are known as inhibitory co-receptors regulating the B cell immune response [3, 4]. CD72, a 45 kDa type II transmembrane glycoprotein, belongs to the C-type lectin superfamily [4]. It is expressed from the pro-B through the mature B cell stage but not on terminally differentiated plasma cells [5, 6]. The protein has a hydrophobic transmembrane domain and a cytoplasmic domain containing two potential ITIM [4]. Ligation of CD72 enhances B cell growth and proliferation [7, 8]. $CD72^{-/-}$ mice exhibit a decrease in the total number of B cells in the spleen and lymph nodes, implying that the proper expression of CD72 is essential for B cell development[2, 4]. Little is known about the contribution of CD72 to B cell development and Ig synthesis, especially in the human system. Recently, the lymphocyte semaphorin CD100/

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Abbreviations: anti-CD40/CD32T: anti-CD40 mAb cross-linked with CD32T · **anti-CD72/CD32T:** anti-CD72 mAb cross-linked with CD32T · **β_2-MG:** β_2-microglobulin · **BSAP:** B cell-specific activator protein · **CD32T:** CD32 transfectant · **CpG ODN:** CpG oligodeoxynucleotide · **ERK:** extracellular signal regulatory kinase · **ITIM:** immunoreceptor tyrosine-based inhibitory motif · **PRDI-BF1:** positive regulatory domain I binding factor 1 · **SAC:** *Staphylococcus aureus* Cowan strain · **XBP-1:** X-box binding protein 1

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Sema4D was identified as the natural ligand for CD72 [9]. Enhanced immune responses have been observed in transgenic mice expressing a secreted form of CD100 [10]. Kikutani's group reported that CD100 plays a nonredundant role in the immune response through a unique mechanism involving switching off the negative signals mediated by CD72 [9, 11].

Ig are produced by plasma cells, which represent the terminal differentiation phase of B cell linage development. The triggering of the B cell Ig receptor by an antigen, by cytokines such as IL-2, IL-6 and IL-10 and by direct cell-to-cell contact, for example the CD27/CD70 interaction between B and T cells, plays an important role in the differentiation of memory B cells into plasma cells [12–15]. Although CD40/CD40 ligand (CD154) interaction is critical for B cell proliferation and Ig synthesis, CD40-mediated B cell signaling results in the suppression of B cell differentiation along the plasma cell pathway [16]. B cell activation, proliferation and differentiation is also controlled by cytoplasmic proteins such as the tyrosine kinases Syk, Lyn, Btk and Blk and nuclear proteins such as B cell-specific activator protein (BSAP) [17], X-box binding protein 1 (XBP-1) [18] and positive regulatory domain I binding factor 1 (PRDI-BF1/Blimp-1) [19]. XBP-1, a member of the CREB/ATF family of transcription factors, is the only transcription factor known to be selectively and specifically required for the terminal differentiation of B lymphocytes into plasma cells. XBP-1-deficient murine B cells fail to become antibody-secreting cells *in vivo* in response to antigen-specific, T cell-independent or -dependent stimuli [18]. Another important molecule involved in the differentiation of memory B cells into plasma cells is Blimp-1 (PRDI-BF1 in humans), which is identified as an inducer of terminal B cell differentiation by repression of c-myc transcription [20]. Our results demonstrate that human CD72 plays a key role in the inhibition of Ig synthesis by negatively controlling naive B cell differentiation into plasma cell through the repression of XBP-1.

Results

Preferential CD72 expression on naive B cells

We studied the phenotype of circulating human B cells using double- or triple-color immunofluorescent staining of purified B cells. Until now, it had been reported that CD72 is expressed throughout B cell differentiation, from the earliest B cell progenitors to mature B cells, but is down-regulated upon terminal differentiation into plasma cells [5]. In circulating human B cells, CD72 was expressed preferentially by the $CD27^-$ naive B cell population (Fig. 1A). In marked contrast, $CD27^+$ memory B cells strongly expressed CD80, as previously described (Fig. 1A) [21]. Peripheral blood B cells were separated into three populations according to the expression of CD27 and IgD: IgD^+CD27^- naive B cells, IgD^+CD27^+ memory B cells (so called IgM memory B cells) and IgD^-CD27^+ memory B cells (so called switched memory B cells) [22]. Our results demonstrated that CD72 is expressed by all naive B cells and by the majority of IgM memory B cells but is detected only at low levels on switched memory B cells (Fig. 1B). In contrast, CD80 was strongly expressed by switched memory B cells and only weakly by IgM memory B cells. CD86 was slightly expressed by switched memory B

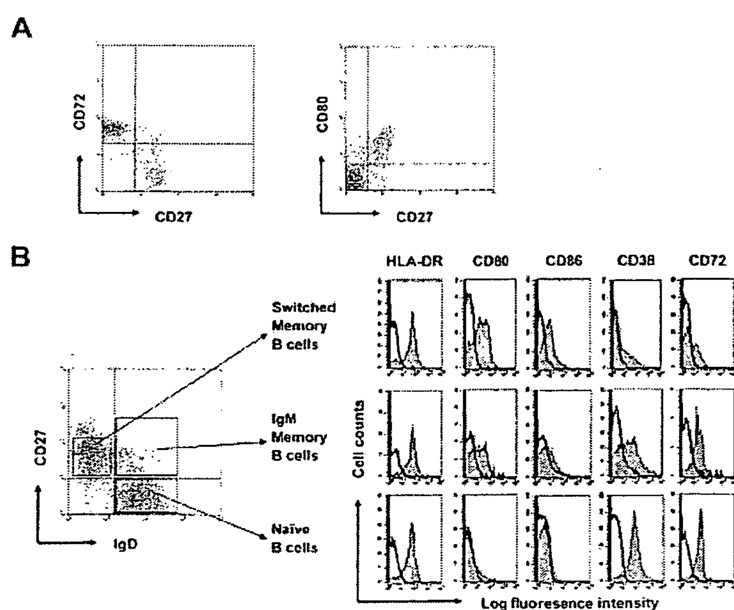

Fig. 1. Expression of surface molecules by human peripheral resting B cells. (A) Purified resting human B cells were stained with anti-CD72-FITC and anti-CD27-biotin followed by streptavidin-PE or with anti-CD80-PE and anti-CD27-FITC and were then evaluated by flow cytometry. (B) Purified resting human B cells were stained with anti-IgD-FITC, anti-CD27-biotin followed by avidin-PerCP and PE-conjugated antibodies against the indicated antigens and were then evaluated by flow cytometry. Resting B cells were divided into three subpopulations according to surface IgD and CD27 expression: IgD^+CD27^- naive B cells (lower right), IgD^+CD27^+ IgM memory B cells (upper right) and IgD^-CD27^+ switched memory B cells (upper left). Dot plot data is displayed with FITC for IgD and PerCP for CD27. Histogram data indicating the fluorescence intensity are displayed with PE for HLA-DR, CD80, CD86, CD38 or CD72 (thin line and closed area) and the isotype control (thick line and open area).

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cells, CD38 was mainly expressed by naive B cells, and all B cell populations expressed HLA-DR equally. These findings demonstrate that naive B cells preferentially express CD72 molecules.

CD72 signaling enhances B cell activation and proliferation

To define the effect of CD72 on human B cell functions, we investigated B cell activation and proliferation in response to CD72 signaling. Purified human B cells stimulated with anti-CD72 mAb cross-linked with CD32 transfectant (anti-CD72/CD32T) showed a modest increase in the expression of CD69 in the presence of *Staphylococcus aureus* Cowan strain (SAC) plus IL-2 or anti-CD40 mAb cross-linked with CD32T (anti-CD40/CD32T). In the presence of CpG oligodeoxynucleotides (CpG ODN), no difference in CD69 expression was observed as a result of the engagement of CD72 with anti-CD72/CD32T (Fig. 2A). It is worth noting that CD27 expression was reduced with anti-CD72/CD32T in the presence of SAC plus IL-2 or anti-CD40/CD32T and not reduced in the presence of CpG ODN (Fig. 2A). Variant tyrosine phosphorylated proteins were mildly augmented by the stimulation with anti-CD72 (Fig. 2B). Immunoprecipitation analysis showed that anti-CD72 engagement clearly enhanced the phosphorylation of Blk, which is a member of the protein tyrosine kinases that associate with the B cell antigen receptor complex (Fig. 2C). B cell proliferation was also enhanced following stimulation with anti-CD72/CD32T in the presence of SAC, SAC plus IL-2, anti-CD40/CD32T or IL-10 plus anti-CD40/CD32T but not in the presence of CpG ODN (Fig. 3). These findings indicate that CD72 signaling strengthens the activation and proliferation of B cells as has been reported elsewhere [7, 8, 23]. However, CD72 signaling reduces the expression of CD27, which plays a crucial role in plasma cell differentiation, and does not influence the CpG pathway.

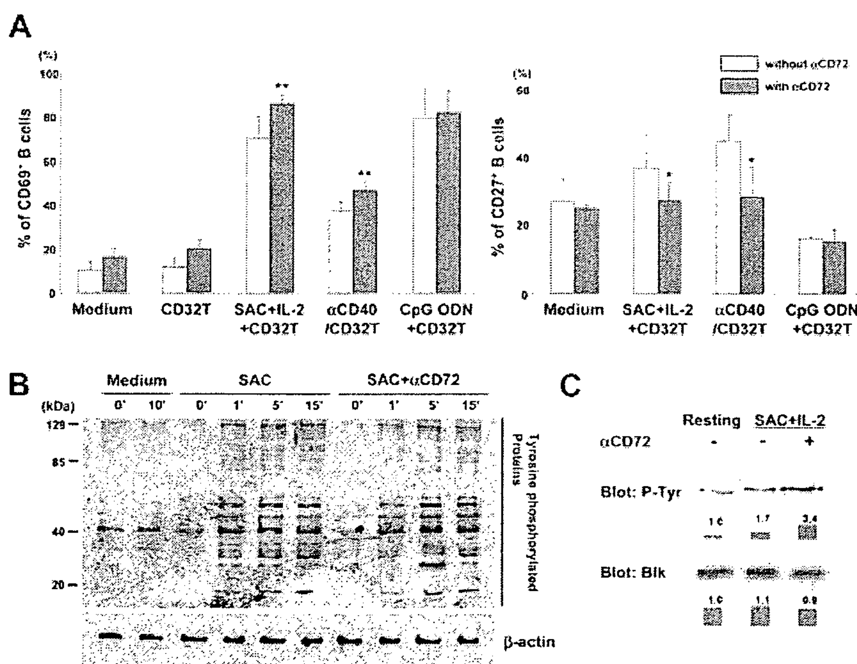

Fig. 2. CD72 stimulation activates human B cells. (A) Purified human B cells were cultured in 96-well round-bottom plates with or without anti-CD72 (5 μg/ml)/CD32T in the presence of SAC (0.01%) plus IL-2 (50 ng/ml), anti-CD40 (1 μg/ml)/CD32T or CpG ODN (2 μg/ml) with a final cell density of 5×10^5/ml in a volume of 200 μl per well for 1 and 3 days at 37°C in a humidified atmosphere with 5% CO_2. "Medium" shows cells incubated with medium alone as a control. CD32Twere added to all wells (with the exception of the medium control) as 20% of the B cell count. Cultured cells were stained with anti-CD20-FITC and anti-CD69-PE at day 1 and with anti-CD20-FITC and anti-CD27-biotin followed by avidin-PE at day 3 and were then evaluated by flow cytometry. Three independent assays were performed, and standard deviations are shown (**$p<0.01$, *$p<0.05$). The values were statistically analyzed by unpaired t-test. (B) Purified human B cells (2×10^6/lane) were pretreated with or without anti-CD72 (5 μg/ml) for 30 min on ice and were stimulated with SAC (0.01%) at 37°C for the indicated time points. Cell lysates were analyzed using immunoblotting with anti-phosphotyrosine Ab and anti-β-actin Ab. The results depicted are representative of three independent experiments. (C) Purified human B cells (2×10^6/lane) were pretreated with or without anti-CD72 (10 μg/ml) for 30 min on ice and were stimulated with SAC (0.01%) at 37°C for 5 min. Cell lysates were immunoprecipitated with anti-Blk Ab and blotted with anti-phosphotyrosine Ab (P-Tyr) or anti-Bkl Ab. Similar results were obtained in another experiment. The fold induction is shown directly below each band.

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