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Lack of Nonfunctional B-cell Receptor Rearrangements in a Patient with Normal B Cell Numbers Despite Partial *RAG1* Deficiency and Atypical SCID/Omenn Syndrome

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Abstract
Introduction A 2.5-month old boy presented with recurrent wheezing, protracted diarrhea, erythrodermia, and failure to thrive.
Methods and Results Laboratory analysis showed lymphocytopenia with severely reduced T-cell numbers but normal numbers of B and NK cells. Serum IgE was increased and the patient had eosinophilia. These presentations are consistent with atypical severe combined immunodeficiency (SCID)/Omenn Syndrome and the diagnosis was confirmed by demonstration of homozygosity for the R841W mutation in the catalytic core of *RAG1*. Comparison of the patient's immunoglobulin heavy chain rearrangements to those of age-matched controls, cord blood, and adults revealed an almost total lack of nonproductive rearrangements (2.7% versus 14.7%, 27.6%, and 19.8% in the controls, respectively) indicating failure to correct out-of-frame rearrangements by a second rearrangement on the homologous chromosome 14.
Conclusion We hypothesize that the R841W mutation causes a malfunction of RAG1 that has differential outcome on V(D)J recombination in B and T cells, as the patient had normal B cell numbers but suffered severe alpha–beta T-cell immunodeficiency.

Keywords Immunodeficiency · RAG · Omenn syndrome · atypical SCID · V(D)J rearrangements

Abbreviations

RAG	recombination activated genes
Ig	immunoglobulin
SCID	severe combined immunodeficiency
BCR	B-cell receptor
TCR	T-cell receptor
OS	Omenn syndrome
PBMCs	peripheral blood mononuclear cells
CDR	complementarity determining region

Case Report

The recombination activated genes 1 and 2 (*RAG1* and *2*) are essential for V(D)J rearrangement of the B- (BCR) and T-cell receptors (TCR) and hence for the development of B and T cells. Patients with mutations in *RAG1* and *2* present with a variety of clinical symptoms. Amorphic mutations cause severe combined immunodeficiency (SCID) with lack of both B and T cells [1]. Hypomorphic mutations leading to the formation of RAG proteins with reduced efficiency caused by amino acid substitutions or premature stop codons have been found in most patients suffering from Omenn syndrome (OS) [2]. OS is characterized by severe immunodeficiency with circulation of oligoclonal, activated T cells. These T cells can be found infiltrating skin and viscera, which causes erythrodermia, hepatosple-

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nomegaly, lymphadenopathy, diarrhea, and failure to thrive. Hyper-IgE is another common clinical finding [2–4]. In the first *RAG*-deficient OS patients described, B cells were almost absent in the periphery and the patients suffered from hypogammaglobulinemia except for hyper-IgE [2]. However, later studies have shown that hypomorphic *RAG* mutations can present without skin symptoms and that normal or only slightly reduced levels of B cells and immunoglobulins may be present [4–7]. Recently, a third phenotype caused by hypomorphic *RAG* mutations was described. This phenotype is characterized by a preponderance of activated gamma–delta T cells, systemic CMV infection, and autoimmune cytopenias [8]. At least 55 different hypomorphic *RAG* mutations (single nucleotide mutations, deletions and duplications) have been described [4–8]; however, no clear correlation between the type of mutation and the clinical presentation has evolved. More cases are needed to clarify these matters.

We here present a boy born to first cousin parents of Palestinian descent. Pregnancy was uneventful, but the boy was hospitalized at day 4 with cyanosis, probably related to feeding. From 2 weeks of age, the patient had chronic cough, coryza, and recurrent wheezing, and these symptoms were followed by diarrhea, erythrodermic nonpruritic eczema, and failure to thrive. He was also treated for several respiratory infections including *Haemophilus influenzae* and *Streptococcus pneumonia*. At 2.5 months of age, the patient was hospitalized again and *Pneumocystis jerovicii*, CMV, *Candida*, and *Escherichia coli* was recovered from the lower airways and *Clostridium difficile* and *Candida* from stools. At this stage, a primary immunodeficiency was suspected. Immunophenotypic analysis showed normal leukocyte counts, but lymphocytopenia caused by much reduced numbers of T cells (Table I) was found. The few T cells showed signs of activation: 42% expressed surface HLA-DR and 42% surface CD25. Twenty-one percent of the T cells used the gamma–delta TCR. Almost all (96%) of the $CD4^+$ T cells were $CD45RA^-RO^+$. NK cell counts were normal. Maternal engraftment was excluded by HLA typing and microsatellite analysis. The clinical presentation and laboratory finding were consistent with OS and the diagnosis was supported by further clinical findings of eosinophilia and hyper-IgE (Table I). It was, however, surprising to find normal concentrations of B cells and normal (or slightly increased) levels of serum IgM, IgG, and IgA (Table I). The ratio of surface immunoglobulin kappa light chain to lambda light chain was 0.57. Antinuclear antibody test was negative.

Sequencing of the *RAG1* and *2* demonstrated homozygosity for two replacement mutations in *RAG1:* G858A (amino acid replacement R249H) and C2633T (R841W) (compared with GenBank accession number M29474). No mutations were found in *RAG2* (identical to M94633). The *RAG1* R249H substitution is a common polymorphism [5]. The father, who showed no signs of immunodeficiency, was homozygous for this mutation, indicating that it was not responsible for the disease. In contrast, only the patient was homozygous for the R841W mutation (both parents were heterozygous carriers). The R841W mutation has previously been found in homozygous form in a child suffering from atypical SCID with CMV infection [8]. This child also had normal immunoglobulin levels and almost normal B-cell numbers (0.2×10^9/L).

Table I Laboratory Data for The Patient at 2.5 months of Age

	Patient (10^9/L)	Laboratory reference values (10^9/L)
Leukocytes	6.6	5.5–17
Neutrofilocytes	1.9	0.9–8.5
Eosinophils	1.3	0.04–0.50
Platelets	464	150–400
Lymphocytes	1.0	1.9–12.4
Lymphocyte subsets		
Total T cells ($CD3^+$)	0.46	3.2–11
Helper T cells ($CD3^+/CD4^+$)	0.32	0.9–4.1
Cytotoxic T cells ($CD3^+/CD8^+$)	0.03	0.1–0.7
B cells ($CD19^+/CD3^-$)	0.39	0.3–1.0
Kappa/Lambda ratio	0.57	0.9–2.0[a]
NK cells ($CD16^+/CD56^+/CD3^-$)	0.82	0.1–1.1
Serum immunoglobulins		
IgM (g/l)	1.24	0.17–1.05
IgG (g/l)	4.0	2.06–6.01
IgA (g/l)	0.09	0.02–0.46
IgE (IU/l)	84.9	<15.0

[a] Reference interval for adults (no units). Values are generally lower in healthy children (mean 1.1 versus 1.5 [9]).

The presence of peripheral B cells prompted us to sequence the BCR rearrangements to analyze the repertoire for possible signs of RAG malfunction in vivo. Immunoglobulin heavy chain rearrangements using the V gene *IGHV3–23* were amplified, cloned, sequenced, and analyzed as previously described [9]. The only modification from the described protocol was that DNA used for polymerase chain reaction (PCR) amplification was purified from full blood or peripheral blood mononuclear cells (PBMCs) without enrichment for memory B cells. Immunoglobulin rearrangements from two age-matched controls, two cord blood samples, and two adults were also sequenced and analyzed using the same protocol.

Seventy-eight unique IgH rearrangements (defined according to [9]) were sequenced from the patient along with 119 unique rearrangements from cord blood, 124 from age-matched controls, and 117 from adults. We only chose to analyze the unmutated sequences, as sequences containing somatic hypermutations have been through the germinal center and therefore do not reflect only the

processes taking place during B-cell development in the bone marrow. A sequence was defined as unmutated if it had less than three mutations in the heavy chain variable (VH) region. This cutoff was chosen because we have previously shown that one to two mutations in the VH region can be explained by *Taq* errors alone [9]. Five rearrangements (6.4%) amplified from the patient were slightly mutated (three to six substitutions in the VH region), one slightly mutated sequence was found in cord blood and two in the age-matched control sequences. From the adult controls 20.5% of the rearrangements had from three to 34 substitutions in the VH region. Only unmutated rearrangements (73 from the patient, 118 from cord blood, 122 from age-matched controls, and 96 from adult controls) were used in the analysis.

The analysis revealed a broad antibody repertoire in the patient including the use of 12 of 32 D gene alleles and the presence of palindromic and N segments (N1 between VH and D: 0–15 nucleotides long, N2 between D and JH: 0–23 nucleotides long). Our method biases amplification of rearrangements using *JH6*, and yet we also identified rearrangements using *JH3*, *JH4*, and *JH5*. When the rearrangements from the patient was compared to rearrangements from the three control groups, no significant differences ($p<0.005$ used because of multiple comparisons) were found in the number of times the different D gene alleles were used (Kruskal–Wallis, $p=0.75$), the number of nucleotides trimmed off the VH ($p=0.16$), DH ($p=0.39$ for 5′ ends and $p=0.09$ for 3′ end) and JH ($p=0.05$) gene segment ends, the lengths of the N segments ($p=0.26$ for N1 and $p=0.03$ for N2), the lengths of P nucleotides ($p=0.09$ 3′ of VH, $p=0.11$ 5′ of D, $p=0.13$ 3′ of D and $p=0.40$ 5′ of JH) or the CDR3 lengths when the JH-gene usage is taken into account ($p=0.18$) (data not shown).

However, a striking observation was that only two of 73 rearrangements (2.7%) from the patient were nonproductive, defined as a rearrangement that broke the normal reading frame of the *JH* gene or had a premature stop codon in an N or D segment. The corresponding numbers were 14.7% from cord blood, 27.6% from age-matched controls, and 19.8% from adults. These differences were statistically significant ($p<0.005$ for all comparisons, Fisher's exact test). Even when restricting the analysis to rearrangements using the same *JH* gene (*JH6*), the difference between the patient and the controls in the fraction of nonproductive rearrangements was statistically significant ($p<0.0001$). Comparison of the three control groups revealed no difference ($p>0.05$, Chi square) and hence the fraction of nonproductive rearrangements appears not to be age-dependent.

D genes can be used in three reading frames. In general, each *D* gene has a hydrophilic and a hydrophobic reading frame along with a reading frame containing one or more stop codons. Predominant use of the hydrophilic reading frame of *D* genes has been claimed to be a sign of selection [10, 11]. In the patient, the majority (55%) of the identified *D* genes were in the hydrophilic reading frame. The use of the three *D* gene reading frames were not different from the distribution of reading frames found in the control sequences ($p=0.4$, Chi square). This suggests that the B cells from the patient have undergone the normal selection processes in the bone marrow.

Discussion

We here describe a patient homozygous for the C2633T mutation in *RAG1*. The mutation gives rise to a substitution of an arginine (R) to a tryptophan (W) residue in position 841. This position is within the core region of the RAG1 protein known to be essential for V(D)J recombination [3, 4]. The mutation has also been described in a compound heterozygous form in a patient with atypical SCID/Omenn Syndrome [4] and in homozygous form in a patient with alpha–beta TCR T-cell lymphocytopenia and CMV infection [8]. Although a formal demonstration of reduced activity of the resulting RAG1 protein is still pending, it is reasonable to assume that this mutation is causative of the immunodeficiency in all three patients. Despite the R841W substitution, these patients all have normal serum immunoglobulin concentrations and (almost) normal levels of B cells. This is in contrast to severely depressed numbers of alpha–beta T cells and clear clinical signs of T-cell immunodeficiency.

To analyze the effect of the mutation on BCR generation, we sequenced VDJ rearrangements from peripheral B cells. We found a broad immunoglobulin repertoire, which did not differ from that of age-matched controls, cord blood samples, and adults with respect to *DH* gene usage, trimming of gene segments, presence of palindromic and N segments, CDR3 length, or selection of hydrophilic reading frames for D segments. A striking observation, however, was that the immunoglobulin rearrangements from the patient were almost always functional. Because B cells cannot develop in the bone marrow without a functional heavy chain VDJ rearrangement assuring signaling through the pre-B-cell receptor [12], nonfunctional rearrangements in the periphery are carried in B cells, which subsequently have acquired a functional VDJ rearrangement on the other chromosome 14. The lack of nonfunctional rearrangements in the patient therefore suggests that the R841W mutation gives rise to a RAG1 protein that prevents generation of a secondary (rescue) rearrangement and thereby deems the cell to death by apoptosis in the bone marrow. The mechanism could involve reduced ability to bind to the DNA or to the other

proteins in the RAG1/RAG2/HGM1 complex or simply be a consequence of reduced enzymatic activity. Hence, if it is envisaged that each B cell only have a limited time frame for recombining the heavy chain gene segments, the mutant RAG1 may lead to a one-shot situation where only pre-B cells producing a functional VDJ rearrangements in the first attempt are able to progress to light chain rearrangement. The deficiency seems to have only limited effect on the generation and function of B cells, whereas it is nearly detrimental to alpha–beta T-cell generation.

A reduced efficiency of RAG1 leading to a one-shot rearrangement situation may explain the elevated ratio of gamma–delta T cells compared to alpha–beta T cells, which is also found in another patient with the RAG1 R841W mutation [8].

In the developing T cell, the delta chain rearranges first followed by the gamma chain and then the beta chain [13]. Alpha chain rearrangement is initiated only after beta chain selection and often relies on multiple, sequential rearrangements to generate a functional gene [13, 14]. A less efficient RAG1 enzyme could lead to a situation where alpha chain rearrangements are rarely initiated or rarely allowed to perform sequential rearrangements leading to failure to produce functional alpha–beta T cells. Gamma–delta T-cell production would be less affected. Likewise, B cells could be less affected than alpha–beta T cells if, for example, light chain rearrangement progresses more efficiently. This is suggested by the relative abundance of B cells carrying lambda light chains (kappa/lambda ratio: 0.57, Table I). The normal kappa/lambda ratio in serum from 0- to 1-year-old infants is 0.65–1.89 [15]. Although surface levels may vary slightly from serum levels, it suggests that the ratio in the patient is normal (or close to normal), and it clearly shows that lambda rearrangements do occur. It has been suggested that in most B cells the kappa light chain rearranges first, followed by lambda light chain rearrangement only if a functional kappa rearrangement fails to be generated or the kappa-deleting element is activated [16]. This would suggest that several light chain rearrangements have occurred in most of the B cells of our patient.

BCRs are generated in the bone marrow, whereas the TCR rearrangements take place in the thymus. Hence, another explanation for the different effect of the found R841W mutation on BCR and TCR rearrangements could be that the expression, translation, degradation, and/or regulation of *RAG1* differ in the two tissues and in B and T cells.

The patient was treated by bone marrow transplantation from an unrelated, HLA-matched donor at the age of 6 months. He obtained complete hematopoietic donor chimerism and remains alive with minor infectious problems 3 years after transplantation.

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